REMARKS

Claims 1-3, and 14-32 were pending. Claims 1-5 and 14-32 have been canceled without prejudice. Applicants reserve the right to prosecute the subject matter of any canceled claims in one or more continuation, continuation-in-part, or divisional applications. New claims 33-42 have been added to more particularly point out and distinctly claim the invention. Support for the new claims may be found in the specification, e.g., at page 20, lines 14-15; page 32, line 15; page 16, line 8; and page 25, lines 14-16.

Applicants respectfully request entry of the amendments and remarks made herein into the file history of the present application. Upon entry of the present amendments, claims 33-42 will be pending and under active consideration.

The Rejections Under 35 U.S.C. § 112, Second Paragraph Should Be Withdrawn

Claims 1-3 and 14-32 were rejected under 35 U.S.C. § 112, second paragraph for indefiniteness. The Office Action asserts that the limitation of an "ETB selective antagonist" in claims 1 and 14-21 was not clearly different from the limitation of an "ETB specific antagonist" in claims 22-32. The Office Action also asserts that the term "small" was not defined as it related to the limitation of a "small molecule inhibitor" in claims 1, 15-18, 21, 22, 27-30 and 32. These rejections are obviated in light of the cancellation of these claims, and Applicants respectfully request that the rejections be withdrawn.

Applicants herein present new claims 33-42, drawn to methods for preventing the initiation, development, or progression of melanoma. Applicants submit that these claims are not indefinite, as detailed *infra*.

Claims 33-42 uniformly claim use of an endothelin B receptor (ETB) specific antagonist. Claims to an ETB specific antagonist are supported in the specification as filed (*see*, e.g., page 20, lines 14-15; page 32, line 15; page 16, line 8; and page 25, lines 14-16).

The present invention as claimed relates to methods for preventing the initiation, development, or progression of melanoma, comprising administering a compound that is a specific antagonist of ETB. In accordance with the invention as claimed and described in the specification, specific antagonists of ETB encompass, *inter alia*, peptide inhibitors, small molecule inhibitors, and antibodies. The specification provides examples of compounds that demonstrate activity as specific ETB antagonists such as BQ788, IRL-

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1038, and RES-701-1 (*e.g.*, page 16, lines 8-13 of the instant specification). ETB specific antagonists may, for example, prevent downregulation of E-cadherin, prevent downregulation of β-catenin, and/or prevent caspase-8 activation (*e.g.*, page 24, lines 26-28, page 48, lines 15-19, and page 49, lines 13-14 of the instant specification). The specification also provides examples of the types of activities that an ETB specific antagonist would be expected to demonstrate and provides numerous assays which could be utilized to identify whether a compound is a specific ETB antagonist (*e.g.*, Sections 5.6.1 and 5.6.2 of the instant specification).

Thus, Applicants submit that claims drawn to use of an "ETB specific antagonist" would be clearly understood by one skilled in the art.

New claims 37, 38, and 42 recite administration of an ETB specific antagonist which may be a "small molecule inhibitor." In opposition to the Office Action's assertion that the term "small" in "small molecule inhibitor" is not definite, Applicants submit that the term "small molecule" was an art-accepted term at the time of filing of the instant application.

All that is required under § 112, 2nd paragraph is that the claims, read in light of the specification, reasonably apprise those skilled in the art of the scope of the inventions. No more is required. Miles Laboratories, Inc. v. Shandon Inc., 997 F. 2d 870 (Fed. Cir. 1993), cert. denied, 510 U.S. 1100 (1994).

As of the filing of the instant application, the term "small molecule" was in common usage in the art. Applicants direct the Office Action's attention to Cho and Juliano, "Macromolecular versus Small Molecule Therapeutics: Drug Discovery, Development and Clinical Considerations", Trends Biotechnol. 1996, 14:153-158 ("Cho"), attached as Exhibit A. The title of Cho's review underlines the distinction of a small molecule as smaller than a macromolecule. Small molecules are less complex than macromolecules such as proteins; thus, small molecules are easier to synthesize chemically, which makes them ideal for drug screening and use as drugs (Cho, page 153, paragraph bridging first and second columns). Cho gives an approximation of typical drug small molecules as having a molecular mass of 300-500 daltons (Cho, page 155, second column, second paragraph). Thus, the art at the time of filing understood the term "small molecule" to mean a simpler organic compound relative to a macromolecule. As the term "small molecule" was understood in the art at the time of filing, Applicants submit that the specification need not define the term "small" in "small molecule inhibitor."

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The Office Action indicated that the Applicants should reformat the Markush groups of claims 2 and 23. Applicants note the suggestion in the Office Action and have reformatted the Markush groups of claims containing Markush groups in accordance with the Office Action's recommendation.

Applicants submit that new claims 33-42 are clear and definite and meet the requirements under Section 112, second paragraph.

The Objection Under 37 C.F.R. § 1.75 Should Be Withdrawn

The Office Action objects to claim 31 and claim 20 as being substantial duplicates. This objection is now moot as these claims have been canceled.

The Rejections Under 35 U.S.C. § 112, First Paragraph Should Be Withdrawn

Claims 1, 2, and 14-32 are rejected under 35 U.S.C. § 112, first paragraph for lack of written description. Essentially, the Office Action asserts that the specification describes a method of treating melanocytes and melanoma but not broadly treating "any cancer comprising the administration of an ETB-specific antagonist." Further, claims 1-3, 14-19, 21-30 and 32 are rejected under 35 U.S.C. § 112, first paragraph for inadequate enablement. Essentially, the Office Action asserts that although the application enables the treatment of cancer associated with upregulated ETB expression, treatment of all cancers is not enabled. Although Applicants do not agree and in no way acquiesce, simply to advance prosecution, pending claims 1-3 and 14-32 are replaced with revised claims 33-42. These claims are fully enabled by the specification as filed. Applicants submit that the cancellation of claims 1-3 and 14-32 renders these rejections moot. Applicants specifically reserve all rights to pursue any subject matter removed from the claims in a subsequent continuation or divisional application.

The Office Action states that one of skill in the art would conclude that the Applicants are in possession of a method of treating melanoma, although the Office Action contends that Applicants are not in possession of a method for broadly treating any cancer.

Applicants note that new claims 33-42 are drawn solely to methods for the prevention of the initiation, development, or progression of melanoma. The specification provides extensive support for using the ETB specific antagonists of the invention for prevention of the initiation, development, or progression of melanoma. The Applicants have shown that ET signaling in melanocytes leads to downregulation of E-cadherin (page

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47, lines 26-29), downregulation of β-catenin and p120^{CTN} (page 49, lines 10-18), increased caspase-8 activity (page 50, lines 26-30), and development of neoplastic characteristics (page 52, lines 5-25), demonstrating the mechanisms by which abnormal ET signaling is involved in the initiation of melanoma development. The Applicants have further shown that ETB specific antagonists can inhibit early events in melanoma progression resulting from ET signaling (*see*, e.g., page 48, line 18; page 49, lines 13-14, and Figures 1C and 2B).

Therefore, Applicants have demonstrated possession of methods of controlling early cellular changes leading to the initiation, development, or progression of melanoma by application of an ETB specific antagonist. As such, Applicants submit that Applicants were in possession of a method for preventing melanoma, using ETB specific antagonists as described in the specification as filed.

The Office Action states that the specification is enabling for methods of treating cancers which express ETB to a greater extent than surrounding tissues. However, the Office Action asserts that the specification is not enabling for methods of treating cancer which do not express ETB to a greater extent than surrounding tissue, or methods of treating cancer which express ETB to a greater extent than surrounding tissue using antisense therapy. Applicants submit, first, that new claims 33-42 do not claim use of antisense therapy. Second, Applicants submit that the specification fully enables one skilled in the art to practice the subject matter of the new claims for the prevention of the initiation, development, or progression of melanoma.

As discussed *supra*, the instant specification demonstrates the effectiveness of ETB specific antagonists for inhibiting early events associated with melanoma development in melanocytes. The specification teaches that the cell membrane protein E-cadherin mediates the adhesion of melanocytes to keratinocytes in the epidermis, and that loss of contact with keratinocytes causes melanocytes to develop melanoma-like characteristics (page 13, line 29 to page 14, line 6). Thus, loss of E-cadherin-associated adhesion is an important step in the progression toward a malignant phenotype. The specification further teaches that the cytoplasmic protein β-catenin is critical for E-cadherin-mediated adhesion, and that disruption of cadherin-catenin function may be integral to the development of diseases relating to cell-cell contact (page 14, lines 9-19). In the specification, the Applicants demonstrate that application of ET-1 to human melanocytes leads to downregulation of E-cadherin (page 47, lines 26-29), downregulation of β-catenin (page 49, lines 10-11), and

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development of neoplastic characteristics (page 52, lines 5-25). The Applicants then demonstrate that application of an ETB-specific antagonist can, at a minimum, block downregulation of E-cadherin (page 48, line 18, and Figure 1C) and β-catenin (page 49, lines 13-14, and Figure 2B). This reveals that an ETB specific antagonist can prevent a non-cancer cell, here a melanocyte, from developing attributes characteristic of the early stages of cancer development. The instant specification also teaches how to use the ETB antagonists as a method of treatment for preventing the initiation, development, or progression of melanoma, as well as providing methods of screening for compounds useful for such prevention. Section 5.5 (pages 34-40) detail the use of ETB antagonists in pharmaceutical formulations including routes of administration, manufacture and use of compositions, effective dosages, and packaging. Section 5.6 (pages 40-47) describe screening assays for compounds useful in the methods of the invention. Therefore, the specification as filed enables one of skill in the art to use the methods of the invention for preventing the initiation, development, or progression of melanoma.

Moreover, Applicants submit for the Examiner's consideration Bagnato et al., Cancer Res. 2004, 64:1436-1443 ("Bagnato"), attached as Exhibit B. Bagnato, published several years subsequent to the filing of the instant application, and citing to the findings detailed in the application, describes the use, as disclosed in the instant specification, of ETB specific antagonists for the prevention of melanoma proliferation in vitro and in vivo. Bagnato demonstrates that ETB specific antagonists are effective not just in melanocytes, but also for the prevention of growth and proliferation in primary and metastatic melanoma, despite downregulation of ETB in these cells. Applicants respectfully assert that the data presented in Bagnato provide evidence that the methods of the invention are enabled for the prevention of malignant melanoma growth in tissues where ETB is not expressed to a greater extent than in surrounding tissue.

Bagnato demonstrates that the ETB specific antagonist BQ788 blocks endothelin-stimulated cellular proliferation, adhesion, migration, and invasion of primary melanoma cells in culture (Bagnato, page 1441, 2nd paragraph). Bagnato further demonstrates that the ETB specific antagonist A-192621 inhibits the growth of metastatic melanoma cells in culture and in vivo following transplantation into nude mice (Bagnato, page 1441, 3rd paragraph). The data in Bagnato support the teachings of the instant specification of the usefulness of ETB antagonists for preventing melanoma initiation, progression, and development. Bagnato further demonstrates that the methods of the invention are

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adequately enabled for preventing the malignant growth of melanoma cells, which show downregulation of ETB and therefore do not express ETB to a greater extent than surrounding tissue. Thus, Applicants submit that the invention as claimed is enabled for methods of treating melanoma cells which do not express ETB to a greater extent than surrounding tissue as well as preventing the initiation, development, or progression of melanoma as claimed.

Applicants submit that the methods of new claims 33-42 have adequate written description and are enabled for preventing the initiation, development, or progression of melanoma, and therefore these claims are in form for allowance.

The Rejections Under 35 U.S.C. § 102 Should Be Withdrawn

Claims 1, 2, 20, 22, 23, and 31 are rejected under 35 U.S.C. § 102(e) as being anticipated by U.S. Patent No. 6,063,911 by Vournakis et al. ("Vournakis"). The Office Action states that Vournakis teaches administration of an endothelin antagonist in combination with poly-beta-1-4-glucosamine for the treatment of cancer and proliferative diseases. This rejection is obviated by the cancellation of these claims.

New claims 33-42 are drawn to methods of administering an ETB specific antagonist for preventing the initiation, development, or progression of melanoma. Applicants submit that these claims are not anticipated by Vournakis, and therefore are in form for allowance.

In order for a reference to anticipate a claim, each and every element of the claim must be disclosed in that one reference. Orthokinetics, Inc. v. Safety Travel Chairs, Inc., 1 U.S.P.Q.2d 1081 (Fed. Cir. 1985). "Anticipation under Section 102 can be found only if a reference shows exactly what is claimed. . . ." Structural Rubber Prod. Co. v. Park Rubber Co., U.S.P.Q. 1264 (Fed. Cir. 1984).

Vournakis discloses the use of ET receptor antagonists for treatment of cancer. Vournakis teaches that Ro61, a compound that antagonizes both the endothelin A (ETA) and endothelin B receptors, can decrease proliferation of B16 murine melanoma cells (Vournakis, column 29, lines 63-67).

Applicants agree with the Office Action statement that Vournakis teaches against claims drawn to treating melanoma. The Office Action notes that the ability of an ETA-specific agonist to reverse the proliferative effect of Ro61 would suggest to one of skill in the art that an ETB-specific antagonist would not decrease proliferation in melanoma cells.

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However, as detailed *infra*, Applicants have demonstrated that ETB antagonists are sufficient to halt ET-mediated signaling in melanoma cells. Therefore, Vournakis teaches against the use of ETB-specific antagonists for the treatment of melanoma.

Further, Vournakis fails to teach or suggest the use of endothelin antagonists to prevent the early events associated with cancer, prior to malignant transformation. Vournakis provides no evidence or suggestion that the application of endothelin antagonists to non-metastatic cells would inhibit the cellular changes associated with the progression to a malignant state. In contrast, the instant specification provides extensive support for the use of ETB antagonists to inhibit the early events associated with the prevention of the initiation, development, or progression of melanoma.

The specification teaches that the cell membrane protein E-cadherin mediates the adhesion of melanocytes to keratinocytes in the epidermis, and that loss of contact with keratinocytes causes melanocytes to develop melanoma-like characteristics (page 13, line 29 to page 14, line 6). Thus, loss of E-cadherin-associated adhesion is an important step in the progression toward a malignant phenotype. The specification further teaches that the cytoplasmic protein β-catenin is critical for E-cadherin-mediated adhesion, and that disruption of cadherin-catenin function may be integral to the development of diseases relating to cell-cell contact (page 14, lines 9-19). In the specification, the Applicants demonstrate that application of ET-1 to human melanocytes leads to downregulation of E-cadherin (page 47, lines 26-29), downregulation of β-catenin (page 49, lines 10-11), and development of neoplastic characteristics (page 52, lines 5-25). The Applicants then demonstrate that application of an ETB-specific antagonist can, at a minimum, block downregulation of E-cadherin (page 48, line 18, and Figure 1C) and β-catenin (page 49, lines 13-14, and Figure 2B). This reveals that an ETB-specific antagonist can prevent a non-cancer cell, here a melanocyte, from developing attributes characteristic of the early stages of cancer development.

The specification clearly identifies the methods of the invention for preventing the initiation, development, or progression of melanoma. Vournakis does not teach prevention of the initiation, development, or progression of cancer or any other proliferative disease using the methods of the invention. Therefore, Vournakis does not anticipate the use of ETB-specific antagonists for preventing the initiation, development, or progression of melanoma.

Vournakis does not meet each and every claim limitation and therefore does not anticipate the claimed invention. Thus, the rejection based on this reference cannot stand and must be withdrawn.

The Rejections Under 35 U.S.C. § 103 Should Be Withdrawn

Claims 1-3 and 14-32 are rejected under 35 U.S.C. § 103(a) as obvious over Kikuchi et al. (Kikuchi, et al., 1996, *Biochem. Biophys. Res. Comm.* 219:734, hereinafter "Kikuchi") in view of Nelson et al. (Nelson, et al., 1996, *Cancer Res.* 56:663, hereinafter "Nelson"). The Office Action states that a skilled artisan would be motivated to administer BQ788 to a patient with a primary melanoma expressing the ETB receptor, based on teachings in Kikuchi to use BQ788 to inhibit the growth of melanoma cells expressing ETB and teachings in Nelson that BQ788 binds and antagonizes ETB in vivo. Applicants submit that the cancellation of these claims obviates this rejection.

More importantly, Applicants further submit that new claims 33-42, relating to methods of administering an ETB specific antagonist for the prevention of the initiation, development, or progression of melanoma, are not obvious in view of Kikuchi combined with Nelson.

Kikuchi demonstrates that primary cutaneous melanoma cells express ETB, and that such cells show increased DNA replication in response to ET-1 treatment. Kikuchi fails to show any effect on cell division following incubation with ET-1, and thus Kikuchi does not demonstrate actual melanoma cell proliferation in response to ET-1. Further, in contrast to the primary melanoma cell lines tested in Kikuchi, metastatic cell lines identified in Kikuchi show downregulation of ETB, and do not respond to ET-1 treatment. This indicates that, contrary to the allegation in the Office Action, that Kikuchi rather suggests that attempting treatment of many melanomas, particularly metastatic melanoma, using a compound that targets the ET-1 pathway would be ineffective.

Kikuchi concludes that "the mitogenic effects of endothelin in human primary melanoma are mainly mediated through ETB receptors" (Kikuchi, abstract). However, Kikuchi fails to show any connection between ET and cancer progression, or a link between ET signaling and pre-metastatic events. In fact, as Kikuchi indicates that ETB is downregulated in metastatic melanoma, a skilled artisan would not conclude that ETB might be involved in melanoma progression.

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In contrast, the instant specification details how ET contributes to cancer progression in melanocytes by causing downregulation of E-cadherin, β-catenin, and p120^{CTN} (Examples 1 and 2, p. 47, line 8 to p. 49, line 21), activation of caspase-8 (Example 3, p. 49, line 23 to p. 51, line 20), alterations in E-cadherin and β-catenin subcellular localization, and development of neoplastic attributes (Example 4, p. 51, line 23 to p. 52, line 25). These examples provide evidence of how the E-cadherin pathway, which stabilizes cellular contact adhesion, is altered in melanoma cells by the ET pathway, thus contributing to melanoma cell malignancy. The present specification teaches that ETB is the primary mediator of ET signaling in melanoma cells, and compounds that modulate ET and ETB are useful therapeutic agents for the treatment of cancers (page 8, lines 20-21; page 11, lines 18-23; page 15, line 9 to page 16, line 6; page 19, line 30 to page 20, line 18; page 22, lines 29-31; page 49, lines 10-14; and Figures 1C and 2B). The disclosure of the instant application, therefore, details the means by which ET contributes in the progression to metastatic disease states, and provides compounds and pharmaceutical compositions to inhibit ET's effect on cancer progression by inhibition of the ETB receptor (page 15, line 9 to page 16, line 6, page 19, line 30 to page 20, line 18). The specification details how selective ETB antagonists are capable of blocking the cascade leading to melanoma development (page 24, line 1 to page 25, line 10; page 22, lines 29-31; page 49, lines 10-14; and Figures 1C and 2B). Kikuchi, by disclosing only an effect of ET on DNA replication in melanoma cells, fails to provide these elements, and cannot be seen to suggest, much less teach, the methods of use of the invention.

Applicants additionally point out that Kikuchi does not suggest that BQ-788 treatment would prevent melanocytes from developing into melanoma cells. There is no suggestion in Kikuchi to use ETB specific antagonists for prevention of the progression of melanocytes or any normal cells to early stages of cancer initiation, development, or progression. The Office Action contends that Kikuchi shows inhibition or melanoma cell growth by BQ-788. However, as noted *supra*, Kikuchi does not show any effect of BQ-788 on cell proliferation. Kikuchi also does not show that treatment of primary melanoma cells with BQ-788 inhibits progression to more advanced stages of cancer. Kikuchi also does not suggest any involvement of the E-cadherin pathway as a mechanism for how ET signaling affects melanoma development. There is no support in Kikuchi for using an ETB specific antagonist to prevent cancer, nor is there support for the use of an ETB specific antagonist in the treatment of any cancers other than a specific stage of primary cutaneous melanoma.

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In contrast, the instant specification provides use of ETB specific antagonists for cancer treatment, and for cancer prevention (page 8, lines 20-21, page 11, lines 18-23, page 15, line 9 to page 16, line 6, page 19, line 30 to page 20, line 18, and page 24, line 1 to page 25, line 10). Therefore, Applicants assert that the invention as claimed is not obvious in view of the teachings in Kikuchi.

Nelson discloses that the ETA receptor-specific antagonist A-127722 inhibits ET-stimulated proliferation of human prostate cancer cells, while BQ788 has no effect (Nelson, abstract, and page 665, first full paragraph). Nelson states on page 665, first full paragraph, that this data indicates that "the effects of ET-1 are mediated through ETA only." Nelson further teaches (page 666, second column, first paragraph) that ^{125}I-labeled ET-1 binding to prostate cancer cell lines demonstrates specific ETA binding only, and that "no ETB binding was demonstrated in any of the human prostate cancer cell lines tested." Nelson then discloses that, although ETB binding sites predominate in non-cancerous prostate epithelium, prostate cancer tissue sections show decreased or no ETB binding sites (page 666, second column, second and third paragraphs). Thus Nelson, like Kikuchi, teaches that ETB is downregulated or absent in transformed versus non-transformed tissue. Therefore, the skilled artisan would not be motivated to use BQ788 or any ETB specific antagonist for the treatment of melanoma, prostate cancer, or any cancer, as both Kikuchi and Nelson teach that ETB is not present in cancer tissue, and Nelson further teaches that ETB specific antagonists are not effective for inhibition of cancer cell proliferation.

Nelson cites to findings published in another reference that BQ788, but not an ETA-specific antagonist, inhibits circulating ^{125}I-ET-1 binding in vivo. Nelson refers to these findings in relation to the function of ETB in the circulation, in normal, non-malignant tissue, as part of a discussion of possible consequences of disruption of the function of ETB in cancer cells. The Office Action suggests that one of skill in the art would be motivated to apply BQ788 to a patient with melanoma based in part on this statement in Nelson. However, the skilled artisan would at most conclude, based on the teachings in Nelson in combination with Kikuchi, that, while ETB is present and functional in normal tissues, and BQ788 may be used in vivo, the absence of ETB function in malignant prostate tissue and the lack of effect of BQ788 on growth in prostate cancer cells suggests that administration of BQ788, or any ETB specific antagonist, would be ineffective for the treatment of cancer.

In view of the foregoing, Applicants submit that the teachings of Kikuchi in view of Nelson do not make obvious the methods of the invention for the initiation, development, or
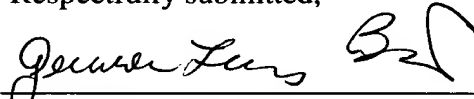
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progression of melanoma. Therefore, new claims 33-42 are not obvious in view of Kikuchi combined with Nelson, and these claims are in condition for allowance.

CONCLUSION

Applicants respectfully request that the amendments and remarks made herein be entered and made of record in the file history of the present application. Withdrawal of the rejections in the previous Office Action and a notice of allowance are earnestly requested. If any issues remain in connection herewith, the Examiner is respectfully invited to telephone the undersigned to discuss the same.

Respectfully submitted,

Date: August 12, 2004

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feature

Macromolecular versus small-molecule therapeutics: drug discovery, development and clinical considerations

Moo J. Cho and Rudy Juliano

Recent advances in biomedical science in general, and molecular biology in particular, have provided a greater understanding of pathogenesis at the molecular and (sub)cellular level. In turn, this has stimulated the development of macromolecular, mechanism-based therapeutic agents, ranging from recombinant proteins, to oligonucleotides, to genes/gene fragments. The factors essential for the successful development of this new class of therapeutic agents are not necessarily the same as those for the development of conventional small organic molecules. This review mentions several issues relating to the development of macromolecular drugs, and emphasizes the key issue of drug transport and delivery.

Throughout most of the history of modern therapeutics, drugs were equated with small organic molecules. Pharmaceutical chemists both in industry and academia synthesized compounds, usually building upon past successes; biologists then screened these compounds for therapeutic potential. Problems of synthesis, scale-up, analytical methodology, formulation and possible pharmacokinetic and pharmacodynamic behavior were all reasonably well understood. Over the past few years, two distinct, but interrelated, trends in drug discovery have emerged that have drastically altered this situation. The advent of the combinatorial library approach to drug synthesis[1-3] has dramatically expanded the types and numbers of compounds available for testing, thus requiring an equally dramatic increase in the speed and precision required of initial screens[4,5]. However, combinatorial chemistry, like traditional drug discovery, relies heavily on drug screening. A second emerging trend makes use of our increasingly sophisticated knowledge about the molecular pathogenesis of disease processes, and involves designing or identifying macromolecules that interact precisely with required targets for therapeutic intervention. In its broadest definition, macromolecular therapeutics encompasses a number of interesting approaches including antisense oligonucleotides, ribozymes, gene therapy, recombinant peptides and monoclonal antibodies. Each of these approaches has its own advantages, as well as problems; however, as we point out below, they share some characteristics that differentiate them from traditional drug development that relies on small organic compounds.

Drug discovery

The classic paradigm of drug discovery has been to synthesize a number of organic compounds, test them in a complex, disease-based, screen, identify a lead compound, undertake further synthesis and then re-initiate the cycle (Fig. 1). Promising compounds would then be introduced into extensive preclinical toxicological testing, and suitable compounds would eventually be clinically tested. Recent innovations have dramatically altered the traditional paradigm. First, drug screening is increasingly target based[6-8]. Rather than initially testing a drug in a complex animal model, rapid, high-throughput screens against defined targets (cloned receptors, enzymes, transcription factors) are used to identify promising candidates. Because of the large numbers of compounds that can be tested this way, data handling and analysis become much more complex, so sophisticated information-handling systems are essential[9]. Increasingly, the insights provided by modern molecular biology, particularly genome research, are being used to design strategies for drug discovery[10]. The identification of genes involved in disease processes has made possible a truly rational approach to therapy, whether it involves small organic drugs or macromolecular drugs.

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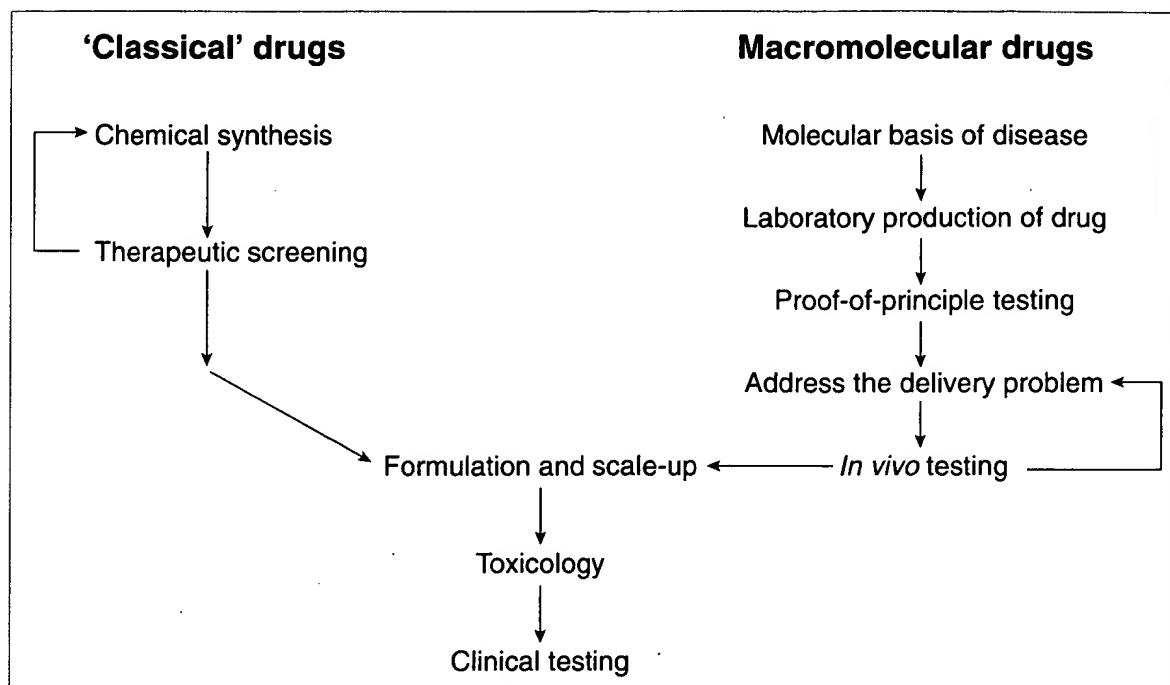
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'Classical' drugs

Chemical synthesis

Therapeutic screening

Macromolecular drugs

Molecular basis of disease

Laboratory production of drug

Proof-of-principle testing

Address the delivery problem

Formulation and scale-up ← *In vivo* testing

Toxicology

Clinical testing

Figure 1

Paradigms for drug discovery. In the discovery phase for 'classic' low molecular weight compounds produced by organic synthesis, a major interactive step occurs between chemical synthesis, as lead compounds are refined and improved, and therapeutic screening (usually in animal models of disease). While scale-up and formulation are important, the transport properties of the drug candidate do not usually represent a major obstacle to further development. Thus, the discovery and development process is relatively simple in structure. For macromolecular drugs, the nature of the therapeutic moiety is defined by an understanding of the pathogenic process. The macromolecule is produced on a laboratory scale, and tested in proof-of-principle experiments where transport and delivery are not an issue (e.g. a therapeutic protein could be directly expressed in cells in culture by transfection with an appropriate vector). For further therapeutic development, the *in vivo* delivery problems must be addressed (e.g. the therapeutic protein may need to be administered by intravenous injection, but the target site is within the cytoplasm of a particular cell type in the body). The iterative process connects the *in vivo* testing of therapeutic efficacy with strategies for macromolecular delivery.

The discovery and development of macromolecular drugs present some unique prospects and problems. For example, many of the recombinant cytokines that are currently undergoing evaluation are highly species-specific. Thus, animal models of efficacy, biodistribution and toxicity are of more limited value than they are for small-molecule drugs, and in-depth human pharmacological studies are required[11].

Using genes as therapeutic agents has challenged the usual pattern of drug development. Genes and vectors are moving from the research laboratory to the stage of clinical testing with less of the extensive preclinical toxicology that has traditionally been associated with the evaluation of low molecular weight drugs[12]. This is not to say that researchers are failing to take suitable precautions to safeguard the welfare of patients. However, the standards concerning the preclinical toxicological evaluation of gene therapy are still evolving[13,14].

Development, production and analysis

The commercial-scale production of recombinant proteins, oligonucleotides and ribozymes, and gene-therapy vectors is a challenging undertaking. The various technologies are currently at different stages of scale-up. The large-scale production of recombinant proteins has made important progress lately[15-17], and

is becoming more routine. The automated production of oligonucleotides on solid supports is now chemically straightforward, but there are still scale-up problems[18]. The least-developed production strategies are those for gene-therapy vectors, but these are evolving quickly[14]. Similarly, analytical and quality-control approaches have evolved to varying degrees. The problems with oligonucleotides are perhaps the least severe, as these are at the low molecular weight extreme of the range of macromolecular drugs, and can be effectively analyzed by capillary electrophoresis, or even by simple HPLC. A variety of analytical technologies and bioassays have typically been used at different stages of the development process of recombinant proteins[19], but a major problem still remains the microheterogeneity of protein preparations[20]. However, the advent of powerful mass-spectrometry techniques for proteins promises to provide precise and quantitative analysis of recombinant proteins. Once again, gene therapy lags substantially behind, relying primarily on complex bioassays to establish quality control.

Drug delivery and transport

The discovery of biotechnology-derived macromolecular therapeutic agents is a more directed

process than tended to be the case for conventional organic drugs (Fig. 1), as a result of our increased understanding of disease pathogenesis at molecular and cellular levels. Thus, the main challenge encountered in development is not so much identifying a bioactive molecule but, rather, how to maintain a therapeutically meaningful concentration of the macromolecule in the vicinity of its target for the desired period of time. The intrinsic difficulty arises from the fact that macromolecules are not readily transported across membranes. In recent years, as increasing numbers of macromolecules with therapeutic potential have appeared in the development pipeline, enormous interest has been directed towards understanding macromolecular transport and delivery. Macromolecular transport *in vivo* requires major biological barriers (Fig. 2) to be overcome between the site of initial administration of a macromolecular drug and its action at a target site within the body. Thus, barriers must be crossed in moving from the bloodstream into tissues, from the extracellular space, across the plasma membrane and into the cytoplasm and, in some cases, from the cytoplasm into the nucleus. We will discuss these barriers in the reverse order.

Cytoplasm to nucleus

The nucleus is enveloped by two layers of membrane that, in places, are fused to each other, creating nuclear pore structures of approximately 70 nm diameter. The pores often occupy as much as 30% of the surface of the nuclear membrane, but their size and number varies depending on the cell type, as well as on the phase of the cell cycle. The pores are spanned by a macromolecular assembly known as the nuclear pore complex[21]. The central aqueous channel of the pore complex mediates the free exchange of small molecules, while regulating the vectorial transport of macromolecules in a sieve-like fashion. Measurements with exogenous tracer molecules suggest an effective pore size of 9–10 nm in diameter.

For endogenous proteins or polynucleotides with a diameter greater than 9–10 nm (approximately 40 kDa), diffusional transport via the pore complex cannot account for entry into the nucleus. Instead, the nuclear pore complex facilitates nuclear localization signal (NLS)-dependent, and ATP-dependent, protein import. The NLS is characterized by a short stretch of positively charged Lys and/or Arg residues (e.g. Thr124-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro135, which encodes part of the large T antigen of SV40). Thus, with covalently attached multiple copies of an NLS sequence, albumin will enter the nucleus when microinjected into the cytoplasm. The exact location of NLS receptors has yet to be firmly established, but potential candidates, collectively referred to as signal-binding proteins, are found not only on the nuclear envelope, but also in the cytosol. This observation supports the possibility that signal recognition by a cytoplasmic protein is followed by transfer of the signal–protein complex to specific receptors on the nuclear envelope and/or the pore complex[22].
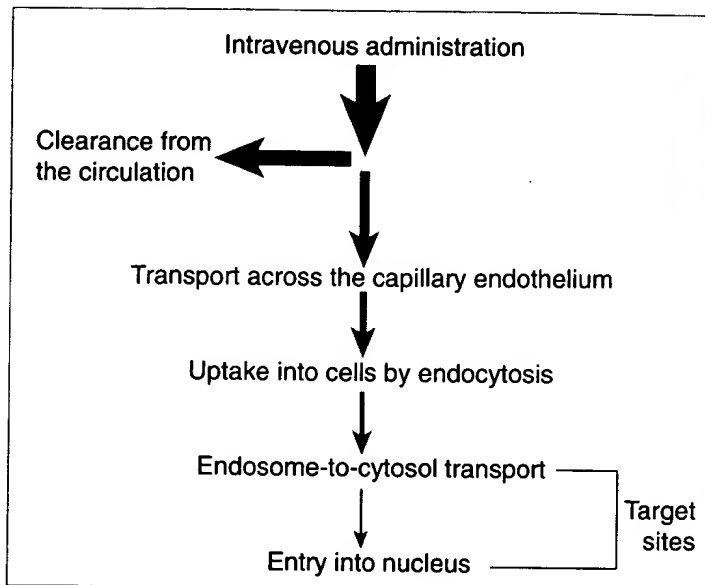

Figure 2

Transport barriers for macromolecular drugs. Subsequent to intravenous administration, a therapeutic macromolecule must overcome several biological barriers to reach its intended target site in the body. Losses occur at each step, as indicated by the arrow sizes. A major goal of macromolecular therapy is to optimize the amount of a therapeutic agent that is delivered to the target.

Despite an incomplete understanding of the NLS-mediated transport mechanism, its utility in delivering exogenous macromolecules to the nucleus will no doubt be actively exploited in the future. Small molecules diffuse almost as rapidly in the cytosol as they do in aqueous solution. Together with the permeability of the nuclear pores, this suggests that delivery of small molecules to the nucleus is not a significant problem, as long as the molecules gain entry to the cytoplasm. For macromolecules too big to enter the nucleus by diffusion, NLS sequences may provide a means of facilitating translocation.

Entry into the cytoplasm

Fick's law, as applied to membrane transport, predicts that, for a given membrane thickness, the solute flux is proportional to the diffusion coefficient of the permeant in the membrane, the partition coefficient between the membrane and surrounding medium, and the concentration gradient across the membrane[23]. For spherical molecules, the diffusion coefficient is inversely proportional to molecular radius, and should not vary by more than 20% for typical organic drug molecules that have molecular masses of 300–500 Da. Therefore, the diffusion coefficient is not generally considered an important variable in traditional drug design. However, as the molecular size increases, the diffusion coefficient decreases quite markedly; according to the Stokes–Einstein equation, the ratio of aqueous diffusion coefficients would be approximately 10:3 for two compounds with molecular masses of 300 Da and 1000 Da, respectively.

Little is known about how size affects membrane partitioning. Amino acids have low n-octanol/water

partition coefficients, and polypeptides have even lower values; this has been attributed to a high level of ground-state hydration[24]. In the case of oligonucleotides, the -OH group in the phosphodiester is a strong acid that is negatively charged at physiological pH. The polyanionic nature of oligonucleotides dramatically reduces their partitioning in membranes. This brief assessment demonstrates how difficult it is for macromolecules to enter the cytoplasm by a passive diffusional process, and highlights a significant difference from small organic molecules.

Endocytosis[25] is part of the constitutive trafficking of cell membrane components; it is also an important pathway for cellular uptake of macromolecular drugs. During membrane invagination, molecules are passively internalized as newly generated membrane vesicles are pinched off and enter the cell. The concentration of ligand on the membrane is a major determinant of the efficiency of internalization. In receptor-mediated endocytosis, a ligand is specifically recognized by its receptor, whereas in adsorptive endocytosis, ligand concentration can occur via nonspecific interactions such as adsorption of cationic macromolecules onto the negatively charged cell membrane. A freely soluble macromolecule with no membrane affinity could enter the cell simply by being part of a volume of fluid pinched off into endocytic vesicles.

Molecules entering cells by endocytosis are sequentially delivered to early and late endosomes, endolysosomes and eventually to lysosomes, where they are degraded[26]. In terms of cytoplasmic delivery, therefore, it is imperative to devise means by which macromolecules can escape from endosomes so that intact molecules can be made available in the cytosol. Along the vesicular transport pathway, the intraluminal pH drops as low as pH 5.0, owing to an ATP-driven H^+ pump in the endosomal membrane[27]. This acidification associated with endosome maturation provides the means by which plant and bacterial toxins[28], and certain viruses[29,30] gain access to the cytosol. In both instances, acid-induced conformational changes in the toxin or viral proteins trigger translocation across the endosomal membrane via a fusion process. Attempts have been made to mimic these strategies for the delivery of macromolecular drugs to their cytoplasmic targets. For example, acid-sensitive liposomes containing various phosphatidylethanolamines have long been promoted for drug delivery to the cytoplasm[31]. In addition, peptides that can undergo a structural change from a random coil at a neutral pH to an amphiphilic helix at pH 5.0 can strongly interact with the membrane to induce pH-dependent membrane fusion[32,33]. Therefore, considerable interest has been shown in using pH-sensitive fusogenic peptides, toxin fragments, surfactants and lipids for the delivery of genes[34], antisense oligonucleotides[35] and proteins[36] to the cytoplasm. Despite some success, many questions remain about the mechanism and efficacy of endosome-to-cytosol delivery of macromolecules.

Gene delivery

A particularly challenging delivery problem is implicit in attempts to perform gene therapy. Vectors based on viruses are beyond the scope of this article, but are reviewed elsewhere[37]. Many workers in the field are seeking to use chemical or physical systems for delivering genes. The most popular approach has involved complexing DNA with polycations such as polylysine, or adsorbing DNA onto preformed cationic liposomes; in some cases, peptides and/or proteins designed to promote cell uptake, release from endosomes, or nuclear targeting, are also included[34,38–41]. Despite their popularity, however, the precise cellular and molecular mechanisms of gene transfer by these polycationic agents have not been firmly established. DNA condensation to small submicron particulates appears not only to protect genes from decomposition by nucleases, but also to promote binding to the negatively charged cell surface. A recent study using various microscopy methods has demonstrated that the complexes are generally quite heterogeneous in size and shape, are taken up by the cell via endocytosis, and require dissociation of the complex before genes can be expressed[41]. Once again, the method of escape of the plasmid from the endosomal compartment to the cytosol is unknown.

Drug distribution in organs and/or tissues

The targeted delivery of a macromolecule from general circulation to a specific cell type within an organ is a further challenge. Proteins[42,43] and oligonucleotides[44,45] are either often unstable and/or clear very rapidly from the bloodstream. For example, recombinant human tissue-type plasminogen activator (tPA) has a circulation half-life of a few minutes and is eliminated from the bloodstream by the liver via mannose-receptor-mediated endocytosis[46], and a 25-mer antisense oligodeoxynucleotide phosphorothioate clears with a half-life of 11 minutes in HIV-infected humans[47]. Approximately 60% of a plasmid DNA expressing chloramphenicol acetyltransferase (CAT) is cleared from the blood of mice within minutes, when injected via the tail vein as a complex with cationic liposomes[48]. As is the case for low molecular weight organic compounds, intravenously administered macromolecules are also cleared mainly by the liver and/or the kidneys[49].

There have been a number of attempts to prolong the circulation life-times of injected macromolecules. When several molecules of polyethylene glycol (PEG) are covalently attached to a protein, its circulation life increases significantly[50]. The chemical conjugation of this flexible hydrophilic polymer provides steric hindrance, blocking interactions between serum opsonins and therapeutic proteins or particulate drug carriers such as liposomes[51–53]. In addition to PEG, human IgG1 (Ref. 54), dextrans[55], ganglioside-G_{M1} (Ref. 56), polysialic acids[57] and other macromolecules[58] have been used, with varying levels of success, to extend the circulatory life of proteins and liposomes.

Once a sustained circulation is achieved, target specificity may be built into a construct by means of

antibodies[59,60] or ligands[61–64] to a specific cell-surface receptor that undergoes endocytosis or transcytosis. For example, an 18-mer peptide nucleic acid antisense to the *rev* gene of HIV-1 has been successfully delivered to the brain of rats when it is conjugated, via a biotin–avidin complex, to an antibody against transferrin receptor[64]. Although conceptually feasible, the successful integration of both long-circulation and organ-specific targeting to a satisfactory level has yet to be demonstrated. In a few instances, however, attaching a targeting ligand appears to be sufficient; for example, a plasmid expressing bacterial CAT was efficiently transferred into the liver of rats by asialoglycoprotein–polylysine receptor-mediated endocytosis; cytoplasmic vesicles were shown to be the main site of persistence of the endocytosed DNA, and constructs remaining in circulation were cleared very rapidly[62].

In summary, one of the most challenging aspects of macromolecular therapeutics will be optimizing the delivery of high molecular weight drugs to their target sites within cells and tissues. It seems likely that, in addition to the strategies that have been explored thus far, this will require innovative approaches.

Clinical issues

Extensive clinical experience has been gained with recombinant proteins[11,65–67]. The number of gene-therapy trials has also increased rapidly over the past two years, with most activity being focused on the treatment of cancer[68]. Several clinical trials of antisense oligonucleotides are also in progress[47]. It seems unlikely that the issues and problems associated with clinical trials of macromolecular drugs will be fundamentally different from those involving traditional drugs. Issues of informed consent, patient safety and welfare, enrollment of sufficient numbers of patients to support the trial design, and data accrual and analysis will continue to be the driving concerns of clinical investigators. One issue that may be of key concern is the possibility that the majority of macromolecular drugs could elicit an immune response: not only might this attenuate the therapeutic benefit, but toxicity might result from activation of immune-system cascades.

Summary – advantages and disadvantages

The relative advantages and disadvantages of low molecular weight and macromolecular drugs are quite clear. Standard drugs that are based on organic chemistry offer relatively straightforward and economical approaches to synthesis and scale-up, and their molecular characteristics and purity can be easily assessed with currently available analytical technology. They can readily be designed to cross membrane barriers and so access their targets on, or within, cells. By contrast, macromolecular drugs present far more complex problems in terms of pharmaceutical-scale production, and they are much more difficult to analyze precisely and quantitatively. In addition, formidable transport and delivery problems are associated with macromolecular therapeutic agents. With all of these disadvantages, one

might wonder why investigators remain so interested in the prospect of using macromolecules as drugs. The answer lies in the potentially exquisite specificity that one can, at least theoretically, attain by using proteins, oligonucleotides or genes as therapeutic agents. The challenge is to convert the potentiality of macromolecular drugs into practical reality.

References
1 Gold, L., Polisky, B., Uhlebeck, O. and Yarus, M. (1995) *Annu. Rev. Biochem.* 64, 763–797
2 Houghten, R. A. (1994) *Curr. Biol.* 4, 564–567
3 Gallop, M. A., Barrett, R. W., Dower, W. J., Fordor, S. P. and Gordon, E. M. (1994) *J. Med. Chem.* 37, 1233–1251
4 Kleinberg, M. L. and Wanke, L. A. (1995) *Am. J. Health Syst. Pharm.* 52, 1323–1336
5 Bevan, P., Ryder, H. and Shaw, I. (1995) *Trends Biotechnol.* 13, 115–121
6 Rosenfeld, R., Vajda, S. and DeLisi, C. (1995) *Annu. Rev. Biophys. Biomol. Struct.* 24, 677–700
7 Caporale, L. H. (1995) *Proc. Natl Acad. Sci. USA* 92, 75–82
8 West, M. L. and Fairlie, D. P. (1995) *Trends Pharmacol. Sci.* 16, 67–75
9 Murray-Rust, P. (1994) *Curr. Opin. Biotechnol.* 5, 648–653
10 Drews, J. (1995) *Arznei.-Forsch.* 45, 934–939
11 Rothig, H. (1994) *Methods Find. Exp. Clin. Pharmacol.* 16, 539–544
12 Vile, R. and Russell, S. J. (1994) *Gene Ther.* 1, 88–98
13 Parker, S. E. *et al.* (1995) *Hum. Gene Ther.* 6, 575–590
14 Norman, J. A., Parker, S. E., Lew, D., Manthorpe, M. and Marquet, M. (1995) *Hum. Gene Ther.* 6, 549–550
15 Hockney, R. C. (1994) *Trends Biotechnol.* 12, 456–463
16 Simonsen, C. C. and McGrogan, M. (1994) *Biologicals* 22, 85–94
17 Houdebine. L. M. (1994) *J. Biotechnol.* 34, 269–287
18 Zon, G. and Geiser, T. G. (1991) *Anti-Cancer Drug Des.* 6, 539–568
19 Anicetti, V. and Hancock, W. S. (1994) *Bioprocess Technol.* 18. 11–36
20 Middaugh, C. R. (1994) *Cytotechnology* 15, 187–194
21 Goldberg, M. W. and Allen, T. D. (1995) *Curr. Opin. Cell Biol.* 7, 301–309
22 Yamasaki, L. and Lanford, R. E. (1982) in *Nuclear Targeting* (Felherr, C. M., ed.), pp. 121–174
23 Flynn, G. L., Yalkowsky, S. H. and Roseman, T. J. (1974) *J. Pharm. Sci.* 63, 479–510
24 Conradi, R. A., Hilgers, A. R., Burton, P. S. and Hester, J. B. (1994) *J. Drug Targeting* 2, 167–171
25 Steinman, R. M., Mellman, I. S., Muller, W. A. and Cohn, Z. A. (1983) *J. Cell Biol.* 96, 1–27
26 Brockman, S. A. and Murphy, R. F. (1993) in *Biological Barriers to Protein Delivery* (Audus, K. L. and Raub, T. J., eds), pp. 51–70, Plenum Press
27 McGraw, T. E. and Maxfield, F. R. (1991) in *Targeting Drug Delivery* (Juliano, R. L., ed.), pp. 11–41, Springer–Verlag
28 Olsnes, S. and Sandvig, K. (1988) in *Immunotoxins* (Frankel, A. E., ed.), pp. 39–73, Kluwer
29 Bron, R., Ortiz, A. and Wilschut, J. (1994) *Biochemistry* 33, 9110–9117
30 White, J. M. (1992) *Science* 258, 917–924
31 Wang, C. Y. and Huang, L. (1987) *Proc. Natl Acad. Sci. USA* 84, 7851–7855
32 Parente, R. A., Nadasdi, L., Subbarao, N. K. and Szoka, F. C., Jr (1990) *Biochemistry* 29, 8713–8719
33 Parente, R. A., Nir, S. and Szoka, F. C., Jr (1990) *Biochemistry* 29, 8720–8728
34 Felgner, J. H. *et al.* (1994) *J. Biol. Chem.* 269, 2550–2561
35 Hughes, J. A., Avrutskaya, A. V., Brouwer, K. L., Wickstrom, E. and Juliano, R. L. (1995) *Pharm. Res.* 12, 817–824
36 Pastan, I. and Fitzgerald, D. (1989) *J. Biol. Chem.* 264, 15157–15160
37 Ali, M., Lemoine, N. R. and Ring, C. J. (1994) *Gene Ther.* 1, 367–384
38 Cotton, M. and Wagner, E. (1993) *Curr. Opin. Biotechnol.* 4, 705–710
39 Behr, J-P. (1994) *Bioconjug. Chem.* 5, 382–389
40 Kavanov, A. V. and Kavanov, V. A. (1995) *Bioconjug. Chem.* 6, 7–20

41 Zabner, J., Fasbender, A. J., Moninger, T., Poellinger, K. A. and Welsh, M. J. (1995) *J. Biol. Chem.* 270, 18997–19007

42 Lemmark, Å., Leslie, R. G. Q. and Werdlin, O. (1987) *Immunol. Today* 8, 353–356

43 Gloff, C. A. and Benet, L. Z. (1990) *Adv. Drug Deliv. Rev.* 4, 359–386

44 Agrawal, S., Temsamani, J., Galbraith, W. and Tang, J. (1995) *Clin. Pharmacokin.* 28, 7–16

45 Srinivasan, S. K. and Iversen, P. (1995) *J. Clin. Lab. Anal.* 9, 129–137

46 Rijken, D. C., Otter, M., Kuiper, J. and van Berkel, T. J. (1990) *Thromb. Res.* (Suppl.), 63–71

47 Zhang, R. *et al.* (1995) *Clin. Pharmacol. Ther.* 58, 44–53

48 Mahato, R. I., Kwabata, K., Takakura, Y. and Hashida, M. (1995) *J. Drug Targeting* 3, 149–157

49 Hashida, M. and Takakura, Y. (1994) *J. Controlled Release* 31, 163–171

50 Katre, N. V. (1993) *Adv. Drug Deliv. Rev.* 10, 91–114

51 Papahadjopoulos, D. (1991) *Proc. Natl Acad. Sci. USA* 88, 11460–11464

52 Yuan, F., Leunig, M., Huang, S. K., Berk, D. A., Papahadjopoulos, D. and Jain, R. K. (1994) *Cancer Res.* 54, 3352–3356

53 Lasic, D. D. (1994) *Angew. Chem. Int. Ed. Engl.* 33, 1685–1698

54 Capon, D. J. *et al.* (1989) *Nature* 337, 525–531

55 Fujita, T., Nishikawa, M., Tamaki, C., Takakura, Y., Hashida, M.

and Sezaki, H. (1992) *J. Pharmacol. Exp. Ther.* 263, 971–978

56 Allen, T. M. and Chonn, A. (1987) *FEBS Lett.* 223, 42–46

57 Gregoriadis, G., McCormack, B., Wang, Z. and Lifely, R. (1993) *FEBS Lett.* 315, 271–276

58 Duncan, R. and Spreafico, F. (1994) *Clin. Pharmacokin.* 27, 290–306

59 Kopecek, J. (1990) *J. Controlled Release* 11, 270–290

60 Allen, T. M. (1995) *Biochim. Biophys. Acta* 1237, 99–108

61 Friden, P. M. *et al.* (1993) *Science* 259, 373–377

62 Chowdhury, N. R., Wu, C. H., Wu, G. Y., Yerneni, P. C., Bommineni, V. R. and Chowdhury, J. R. (1993) *J. Biol. Chem.* 268, 11265–11271

63 Remy, J-S., Kichler, A., Mordvinov, V., Schuber, F. and Behr, J-P. (1995) *Proc. Natl Acad. Sci. USA* 92, 1744–1748

64 Pardridge, W. M., Boado, R. J. and Kang, Y-S. (1995) *Proc. Natl Acad. Sci. USA* 92, 5592–5596

65 White, G. C. (1994) *Ann. Hematol.* 68 (Suppl. 3), S7–S8

66 Demetri, G. D. (1995) *Chest* 107 (Suppl. 6), 255S–260S

67 Felser, J. M. (1994) *Ann. NY Acad. Sci.* 730, 235–242

68 Fenton, R. T., Sznol, M., Luster, D. G., Taub, D. D. and Longo, D. L. (1995) *Hum. Gene Ther.* 6, 87–106

reviews

Engineering challenges in cell-encapsulation technology

Clark K. Colton

The use of implantable immunoisolation devices, in which the tissue is protected from immune rejection by enclosure within a semipermeable membrane or encapsulant, is one approach in the development of cell therapies. However, further research is required in the areas of: tissue supply from primary or cell-culture sources; maintenance of cell viability and function, its relationship to device design, and the role of, and factors affecting, oxygen-supply limitations; and, protection from immune rejection, especially in view of the mechanisms thought to operate in the presence of a semipermeable membrane, the properties of that membrane, and the implications for biology and device design.

Cell therapy involving the transplantation of cells or tissues with specific differentiated functions shows potential in the treatment of human disease. However, the need for immunosuppressive drugs may lead to a variety of serious side effects[1,2]. One approach to minimizing or eliminating systemic immunosuppression is immunoisolation[3], in which the transplanted tissue is enclosed in a semipermeable membrane in order to protect it from immune rejection, thereby creating

what has been termed an implantable biohybrid artificial organ. Devices of this type are under study for the treatment of a variety of diseases, including secretion of insulin in diabetes[4-6], factor IX in hemophilia B (Refs 7,8), human growth factor in dwarfism[9], erythropoietin in anemia[10], as well as for the treatment of kidney failure[11,12], immunodeficiencies[13], and pituitary[14] and parathyroid[15] problems. Potential targets for treatment of disorders in the central nervous system (CNS) include chronic pain[16,17], and neurodegenerative disorders such as Parkinson's disease[18-22], Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis[23]. Treatment of liver failure has

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Endothelin B Receptor Blockade Inhibits Dynamics of Cell Interactions and Communications in Melanoma Cell Progression

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ABSTRACT

Phenotypic and genotypic analyses of cutaneous melanoma have identified the endothelin B receptor (ET$_B$R) as tumor progression marker, thus representing a potential therapeutic target. Here, we demonstrate that activation of ET$_B$R by endothelin-1 (ET-1) and ET-3 leads to loss of expression of the cell adhesion molecule E-cadherin and associated catenin proteins and gain of N-cadherin expression. Exposure of melanoma cells to ET-1 leads to a 60% inhibition in intercellular communication by inducing phosphorylation of gap junctional protein connexin 43. Additionally, activation of the ET$_B$R pathway increases $\alpha_\nu\beta_3$ and $\alpha_2\beta_1$ integrin expression and matrix metalloproteinase (MMP)-2 and MMP-9, membrane type-1-MMP activation, and tissue inhibitor MMP-2 secretion. The ET$_B$R pathway results into the downstream activation of focal adhesion kinase and extracellular signal-regulated kinase 1/2 signaling pathways, which lead to enhanced cell proliferation, adhesion, migration, and MMP-dependent invasion. The small molecule A-192621, an orally bioavailable nonpeptide ET$_B$R antagonist, significantly inhibits melanoma growth in nude mice. These findings demonstrate that ET-1 and ET-3 through ET$_B$R activation trigger signaling pathways involved in events associated with disruption of normal host-tumor interactions and progression of cutaneous melanoma. Pharmacological interruption of ET$_B$R signaling may represent a novel therapeutic strategy in the treatment of this malignancy.

INTRODUCTION

Mouse genetics and molecular profiling of human tissues have identified pathways relevant to melanocyte migration and differentiation as well as to melanoma progression. The study of melanocyte development in knock-out mice has provided insight into the functions of endothelins (ETs) and their receptors in this cell lineage (1–4). The ET family of molecules comprises three isopeptides, ET-1, ET-2, and ET-3, of 21 amino acids that bind to two highly homologous G-protein-coupled receptors, ET$_A$ receptor (ET$_A$R) and ET$_B$ receptor (ET$_B$R), which mediate a variety of physiological activity in different cell types (3). In the adult, ET-1 secreted by keratinocytes stimulates proliferation, chemotaxis, and pigment production in melanocytes (5, 6) and plays a central role in UV-B light-induced pigmentation (7, 8). Increasing evidence points to ETs as relevant mediators in tumor progression in a variety of malignancies (9). Melanoma cells express both ET$_A$R and ET$_B$R (10). Gene expression analysis (11) and immunophenotyping of human cutaneous melanoma (12) have recently identified ET$_B$R as critical in the progression of this malignancy. Through the same receptor, ET-1 acts as antiapoptotic factor for melanoma cells and melanocytes (13). Thus, ET$_B$R blockade by the ET$_B$R peptide antagonist BQ788 resulted in growth inhibition and death of melanoma cells *in vivo* and *in vitro* (14). Although these

studies define a relevant role of the ET-1/ET$_B$R pathway in the biology of melanocytic tumors, the molecular events underlying this activity have not been investigated. Early melanoma growth is the result of disrupted intercellular homeostatic regulation (15). Once this balance is lost and malignant transformation has occurred, microenvironmental factors such as cell adherence to extracellular matrix, host-tumor interactions, degradation of matrix components, migration, and invasion became essential for the tumor progression to the metastatic phenotype (15). Changes in cadherin, connexin (Cx), matrix metalloproteinase (MMP), and integrin expression have emerged as key factors in melanoma progression (16–17). Therefore we analyze the role played by ETs and ET$_B$R in melanoma cell proliferation, cell-cell adhesion and communication, migration, tumor proteinase activation, and invasion. Because activation of focal adhesion kinase (FAK) and of extracellular signal-regulated kinase (Erk) are essential molecular signaling in melanoma progression (18–20), we analyzed the effect of ETs on these ET$_B$R-mediated signal transduction pathways. In view of the availability of orally active nonpeptide ET$_B$R antagonists (9, 21), which are more suitable in clinical setting, we use these compounds to assess their antitumor activity *in vivo*.

MATERIALS AND METHODS

Cells. The human cutaneous melanoma cell line 1007 established from a primary lesion was a generous gift of Dr. G. Parmiani (National Cancer Institute, Milan, Italy). The melanoma cell lines SK-Mel28 (American Type Culture Collection, Rockville, MD) M10 and Mel120 were derived from a metastatic melanoma (22). Cells were grown in RPMI 1640 containing 10% FCS. All culture reagents were from Invitrogen (Paisley, Scotland, United Kingdom). Melanoma cells were starved for 24 h in serum-free medium and then incubated for indicated times with ET-1 and ET-3 (Peninsula Laboratories, Belmont, CA). When the effects of the antagonists [BQ123 and BQ788 (Peninsula Laboratories) and A192621 (Abbott Laboratories, Abbott Park, IL)] were studied, they were added 15 min before agonist. Pretreatment of cells with PD98059 (10 μM; Calbiochem-Novabiochem Corporation, San Diego, CA) or Ilomastat (10 μM; Chemicon International, Temecula, CA) was performed for 20 min before the addition of ET-1 or ET-3.

Reverse Transcription-PCR. Total RNA was prepared using the TRIzol reagent (Invitrogen) as recommended. The reverse transcription-PCR was performed using a SUPERSCRIPT One-Step RT-PCR System (Invitrogen). The primer sets used were 5'-TGCTCCTGCTCGTCCCTGATGGATAAA-GAG-3' and 5'-GGTCACATAACGCTCTCTGGAGGGCTT-3' for ET-1, 5'-4 CACTGGTTGGATGTGTAATC-3' and 5'-GGAGATCAATGACCA-CATAG-3' for ET$_A$R, 5'-TCAACACGGTGGTGTCCTGC-3' and 5'-ACT-GAATAGCCACCAATCTT-3 for ET$_B$R, 5'-AACAGGATGGCTGAAG-GTGA-3' and 5'-AAAATCCAAGCCCTTTGCTG-3' for E-cadherin, and 5'-TTCCAGCAGCCCTACGACCAG-3' and 5'-GCCTTTCCCACTAGTCT-CATC-3' for *Snail*. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control and the primer sets used were 5'-TGAAG-GTCGGTGTCAACGGA-3' and 5'-GATGGCATGGACTGTGGTCAT-3'. The cDNA was amplified for 35 cycles of a denaturation step at 94°C for 1 min; a primer annealing step at 64°C (E-cadherin), 55°C (*Snail*), and 54°C (ET-1, ET$_A$R, ET$_B$R, and GAPDH) for 30 s; and an extension step at 72°C for 1 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide.

Northern Blotting. Total RNA from 1007 cells was separated on 2% denaturing formaldehyde agarose gel (15 μg RNA/lane) and transferred to a nylon membrane. The membranes were hybridized in the QuickHyb hybrid-

ization solution (Stratagene, La Jolla, CA). The cDNA probe used for analysis of the E-cadherin, *Snail*, and GAPDH mRNA was prepared using reverse transcription-PCR products. Probes were labeled with [α-^{32}P]dCTP using a random primer oligolabeling kit (Amersham Biosciences, Little Chalfont Buckinghamshire, United Kingdom). Densitometric scanning was performed with a Mustek MFS-6000CX apparatus, and the data were analyzed with Phoretix 1D software and normalized to those of GAPDH.

Western Blotting. Conditioned medium and protein extracts were separated by SDS-PAGE and revealed by Western blot using antibodies (Abs) to MMP-2 and MMP-9 (NeoMarkers, Fremont, CA), tissue inhibitor metalloproteinase (TIMP)-2, membrane type 1 (MT1)-MMP (Chemicon), phospho-ERK1/2 (Cell Signaling Technology, Inc., Beverly, MA), ERK1/2 (Cell Signaling Technology), α$_v$, α$_2$, β$_1$, β$_3$ (Chemicon), E-cadherin, N-cadherin, β-catenin, p120-catenin, Cx43 (BD Transduction Laboratories, Heidelberg, Germany), and β-actin (Oncogene Research Products, Boston, MA). Blots were developed with an enhanced chemiluminescence detection system kit (Amersham Biosciences).

Gelatin Zymography. The 1007 and M10 cell supernatants were electrophoresed for analysis in 9% SDS-PAGE gels containing 1 mg/ml gelatin. The gels were washed for 30 min at 22°C in 2.5% Triton X-100 and then incubated in 50 mM Tris (pH 7.6), 1 mM ZnCl$_2$, and 5 mM CaCl$_2$ for 18 h at 37°C. After incubation, the gels were stained with 0.2% Coomassie Blue. Enzyme-digested regions were identified as white bands on a blue background. Molecular sizes were determined from the mobility, using gelatin zymography standards (Chemicon).

Immunoprecipitation and SDS-PAGE. Serum-starved 1007 melanoma cells were treated with ET-1 and ET-3 for different times. Lysates were immunoprecipitated with anti-FAK (Upstate Biotechnology) or anti-β-catenin or anti-p120-catenin. The blots were incubated for 1 h with antiphosphotyrosine mAb (PY20; BD Transduction Laboratories) or with anti-FAK (Upstate Biotechnology) and developed with an enhanced chemiluminescence system.

Flow Cytometry Analysis. Serum-starved 1007 melanoma cells were treated with ET-1 and ET-3 (10 nM) for 24 h and incubated with primary Ab to α$_1$, α$_2$, α$_4$, α$_6$, α$_v$, β$_1$, β$_3$, β$_6$, or α$_v$β$_3$ and α$_2$β$_1$ (Chemicon) for 40 min at 4°C. FITC-conjugated secondary Ab (Chemicon) was applied to the cell for 30 min at 4°C. Labeled cells were scanned on a FACScan cytometer (Becton Dickson).

ELISA. MMP-2 and TIMP-2 levels in conditioned medium were measured using a Biotrak Human MMP-2 Elisa kit (Amersham Biosciences) and a human TIMP-2 Immunoassay Kit (Chemicon), respectively. TIMP-2 expression was measured by an ELISA capable of recognizing TIMP-2 complexes with active MMP-2. The range of detection of the assays was of 1.5–24 ng/ml for MMP-2 and 20–320 ng/ml for TIMP-2. The experiments were performed in duplicate and repeated three times.

Scrape Loading/Dye Coupling. Levels of gap junction intercellular communication (GJIC) in control and treated cultures were determined using the scrape-loading/dye transfer (SL/DT) technique, applying a mixture of fluorescent dyes: 0.5% lucifer yellow (LY; Sigma) and 0.5% rhodamine-dextran (Molecular Probes, Eugene, OR). 1007 and M10 cells, cultured as described previously, were washed thoroughly with PBS in which Ca^{2+} was omitted to prevent uncoupling of the cells due to high Ca^{2+}. The mixture was added to the cells, and scrape loading was performed applying two or three cuts on cell monolayer with a razor blade. The dye mixture was rinsed away 1 min after the scrape. Cells were washed three times with PBS and fixed with 4% paraformaldehyde, and cells stained with LY alone and or with rhodamine-dextran were detected by fluorescence emission with an inverted fluorescent microscope equipped with a camera and counted. Junctional permeability was measured after the scrape by taking five successive images per trial. Cells that received the LY from the scrape-loaded cells, excluding the rhodamine-dextran-stained cells, were considered communicating. GJIC capacity was expressed as percentage of the control.

Cell Proliferation Assay. [^{3}H]thymidine incorporation was measured as described previously (23). Responses to all agents were assayed in sextuplicate, and results were expressed as means of three separate experiments.

Adhesion Assay. Serum-starved cells were treated with ET-1 or ET-3 in the absence or in the presence of antagonists for 24 h and labeled by incubation with ^{51}Cr-labeled sodium chromate (50 μCi for 10^6 cells; DuPont, New England Nuclear Research Products, Wilmington, DE) for 1 h at 37°C. As indicated, cells were incubated with Abs to α$_2$β$_1$ and α$_v$β$_3$ subunits (10 μg/ml;

Chemicon) for 2 h at 37°C with gentle shaking. Labeled cells (5 × 10^4) were plated in 96-well plates precoated with type I collagen (10 μg/ml; BD Transduction Laboratories) for 120 min at 37°C. The percentage of cell adhesion was calculated as follows: cpm adherent cells/cpm adherent + cpm nonadherent cells × 100. The assay was performed in sextuplicate, and results were expressed as means of three separate experiments.

Chemotaxis and Chemoinvasion Assay. Chemotaxis and chemoinvasion were assessed with a 48-well modified Boyden chamber (NeuroProbe, Pleasanton, CA) as described previously (24). After 4 (chemotaxis) or 6 h (chemoinvasion) of incubation at 37°C, the filters were removed and stained with Diff-Quick (Merz-Dade, Dudingen, Switzerland), and the migrated cells in 10 high-power fields were counted. Each experimental point was analyzed in triplicate. Pretreatment of cells with Abs to α$_v$β$_3$ and α$_2$β$_1$ (Chemicon) was performed for 60 min at 37°C before the chemotaxis assay.

M10 Melanoma Xenografts. Female athymic (nu$^+$/nu$^+$) mice, 4–6 weeks of age (Charles River Laboratories, Milan, Italy), were handled according to the institutional guidelines under the control of the Italian Ministry of Health. Mice received s.c. injection on one flank with 1.5 × 10^6 viable M10 cells expressing ET$_B$R. The mice were randomized in groups ($n = 10$) to receive treatment i.p. for 21 days with two different doses of A-192621 (10 mg/kg/day and 20 mg/kg/day), and controls received injection of 200 μl of drug vehicle (0.25 N NaHCO$_3$). The treatments were started 7 days after the xenograft, when the tumor was palpable (~0.03 cm^3). Each experiment was repeated three times. Tumor size was measured with calipers and was calculated using the formula π/6 × larger diameter × (smaller diameter)^2.

Statistical Analysis. Statistical evaluations of data were made by the two-sided Student's test and by two-way ANOVA as appropriate.

RESULTS

ET-1 and ET-3 Alter Cell Adhesion Molecule Expression through ET$_B$R. ET-mediated activities were assessed on the primary melanoma 1007 cells and in the metastatic cell lines M10, SK Mel 28, and Mel 120, which express ET$_A$R and ET$_B$R mRNA but no ET-1 mRNA (Fig. 1A). The tumor suppressor E-cadherin, which is often down-regulated in most melanoma (25), has recently been shown to be modulated by ET-1 (26). In 1007 and M10 cells, ETs induced E-cadherin down-regulation upon 24 h of treatment (Fig. 1B). Because the catenins are cytoplasmic proteins that bind E-cadherin and are critical for cellular adhesion, we investigated the effects of ETs on β-catenin and p120-catenin. In both melanoma cell lines, ET-1 and ET-3 stimulation for 24 h decreased the protein levels of β- and p120-catenin paralleling those of E-cadherin down-regulation (Fig. 1B). The shift in cadherin profile from E- to N-isoform during melanoma progression has been described both *in vitro* and *in vivo*, as a mechanism that not only frees melanoma cells from control by keratinocytes but also provides new adhesion properties enhancing the malignant phenotype (16). In 1007 and M10 melanoma cells, we observed that ET-1- and ET-3-induced progressive loss of E-cadherin was concomitant with gain of N-cadherin expression (Fig. 1B), which may enable melanoma cells to interact with N-cadherin-positive neighboring melanoma cells, fibroblasts, and endothelial cells. BQ788, a specific ET$_B$R antagonist, reversed these effects, indicating that ET$_B$R activation is required for these responses (Fig. 1B). The transcription factor *Snail*, a master regulator of epithelial to mesenchymal transition, has recently been found to repress E-cadherin expression. Screening of a panel of melanoma cells from different progression stages revealed that the expression of *Snail* negatively correlates with expression of E-cadherin (15, 27). By Northern blot analysis, we demonstrated that ET-treatment of 1007 cells induced a significant increase in the expression of *Snail* mRNA that closely correlated with down-regulation of E-cadherin at mRNA and protein levels, suggesting that down-regulation of E-cadherin protein involved transcriptional mechanisms (Fig. 1C). Tyrosine phosphorylation of catenin proteins may result in a reduced interaction with both
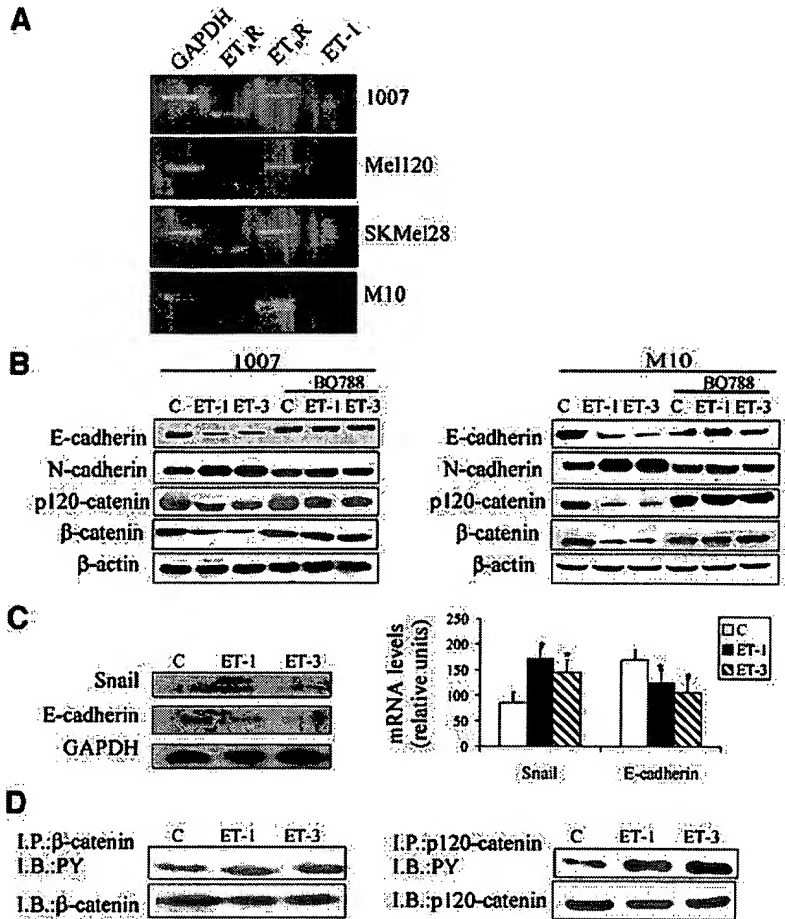
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A

Fig. 1. *A*, ET$_A$R and ET$_B$R mRNAs detected by reverse transcription-PCR in melanoma cell lines. PCR products of 367 bp for ET$_A$R, 529 bp for ET$_B$R, and 533 bp for GAPDH are shown as visualized by ethidium bromide. All of the melanoma cell lines tested, 1007, M10, SKMel 28, and Mel120, expressed mRNA for ET$_A$R and ET$_B$R but no ET-1 mRNA. *B*, down-regulation of E-cadherin and catenin proteins and up-regulation of N-cadherin by ET-1 and ET-3 in melanoma cells. Serum-starved 1007 and M10 cells were stimulated with 10 nM ET-1 or ET-3 for 24 h in the absence or presence of 100 nM BQ788. Lysates were immunoblotted with Abs to E-cadherin, N-cadherin, β-catenin, or p120-catenin and for internal control Ab to β-actin. *C*, Snail mRNA levels expression inversely correlate with E-cadherin after ET-treatment. Snail and E-cadherin mRNA were analyzed by Northern blotting in 1007 cells treated with ET-1 or ET-3 (10 nM) for 6 h. GAPDH mRNA expression levels were used as a loading control. The relative density of mRNA content from *C* was statistically analyzed and represents the average value of three independent Northern blots ± SD; *, *P* < 0.001 compared with control. *D*, ET-1 and ET-3 induce the tyrosine phosphorylation of catenin proteins. Serum-starved 1007 cells were incubated for 5 min with 10 nM ET-1 or ET-3. Cell lysates were immunoprecipitated with anti-β-catenin and anti-p120-catenin and then immunoblotted with antiphosphotyrosine. The membranes were reprobed with the specific anti-β- and p120-catenin to ensure equal amounts of proteins.

E-cadherin and actin-cytoskeleton (28). In this context, we observed that in serum-starved 1007 cells, both isopeptide ET-1 and ET-3 (10 nM) induced an increase in the tyrosine phosphorylation of β-catenin and p120-catenin that contributes to loss of functional cell adhesions (Fig. 1*D*).

Intercellular Communication Is Impaired by ET-1 and ET-3 through ET$_B$R Binding. Loss of GJIC has been shown to occur during melanoma progression. We studied the effects of ETs on GJIC using SL/DT methods in 1007 and M10 cells. For the evaluation of GJIC function, only cells stained with LY, excluding the artificially damaged rhodamine-dextran-stained cells, were counted. Addition of 10 nM ET-1 resulted in a transient and time-dependent reduction in GJIC. Intercellular transfer of LY was still detectable 5 min after ET-1 addition, whereas after 30 min, the dye remained confined to the wounded cells and GJIC were inhibited by 60–70% with respect to the control. GJIC of melanoma cells returned to basal level within 1 h (Fig. 2*A*). To determine whether ET$_B$R was responsible for ET-induced disruption of GJIC, the ET$_B$R antagonist was used in SL/DT experiments. ET-1- and ET-3-induced dye transfer inhibition was completely prevented by BQ788 (Fig. 2*B*). Gap junction formation requires Cx molecules aligning in hemi-channels. Both melanocytes and keratinocytes express Cx43 in coculture (25). The phosphorylation of Cx43 is believed to be casually linked with disruption of GJIC (29). Recent evidence shows that ET-1 decreases GJIC in ovarian carcinoma cells by inducing Cx43 phosphorylation (30). Therefore to evaluate the effect of ETs on Cx43 expression in melanoma cells, 1007 and M10 cells were exposed to ET-1 and ET-3 (10 nM). Non-phosphorylated Cx43 was detected in untreated 1007 and M10 cells. ETs significantly induced increase in electrophoretic mobility shift of

Cx43 (Fig. 2*C*). Exposure of melanoma cells to 10 nM ET-1 and ET-3 resulted in a rapid induction of two phosphorylated species that we refer to collectively as Cx43-P (Fig. 2*C*). BQ788 blocked this effect, indicating that ET$_B$R activation results into phosphorylation of Cx43 and disruption of GJIC.

ET-1 and ET-3 Induce Secretion and Activation of MMPs through ET$_B$R. A critical step during tumor invasion is the degradation of the extracellular matrix (ECM) by MMPs. To identify the role of ETs on the activation status of MMP-2 and MMP-9, conditioned media of 1007 and M10 cells were analyzed by gelatin zymography. When 10 nM ET-1 and ET-3 was added, zymography showed that both melanoma cell lines secreted high levels of gelatinolytic proteases corresponding to the active forms of MMP-2 and MMP-9 (Fig. 3*A*). These results were confirmed by Western blotting, demonstrating that treatment with ET-1 and ET-3 induced an increased secretion of both latent and active forms of MMP-2 and MMP-9 in the conditioned medium of 1007 cells as compared with untreated cells (Fig. 3*B*). MT1-MMP is a transmembrane MMP known to bind and active MMP-2 at the cell surface (31). ET-1 and ET-3 enhanced expression of both the latent MT1-MMP and to an even greater extent, the activated form of MT1-MMP. Recent studies have demonstrated that activation of pro-MMP-2 by MT1-MMP depends upon the presence of critical amounts of TIMP-2, which is required for the formation of the ternary complex that leads to the activation of MMP-2 (32). Both ET-1 and ET-3 induced a significant increase of TIMP-2 expression as demonstrated by Western blotting (Fig. 3*B*). By ELISA, we confirmed that both isopeptides were capable of eliciting MMP-2 and TIMP-2 secretion in the conditioned medium of 1007 cells. Addition of BQ788 completely blocked the ET-induced conversion of

A

Fig. 2. ETs induce GJIC inhibition in melanoma cells. *A*, serum-starved 1007 and M10 cells were treated with 10 nM ET-1 for up to 60 min, and GJIC capacity was assayed by SL/DT method. Gap junction function was evaluated by analyzing net transfer of LY, excluding dextran-stained cells, as described in "Materials and Methods." The data, reported as the relative percentage of the control, represent the average value of three different assays each performed in triplicate samples ± SD; *, $P < 0.005$ compared with control. *B*, serum-starved 1007 and M10 cells were incubated for 30 min with ET-1 or ET-3 (10 nM) in the absence or in presence of BQ 788 (100 nM). Photographs show a representative experiment of SL/DT assay performed on 1007 cells; *bar* = 100 µm. The data, reported as the relative percentage of the control, represent the average value of three different assays each performed in triplicate ± SD; *, $P < 0.005$ compared with control; **, $P < 0.0001$ compared with ET-1 or ET-3. *C*, ET-1 and ET-3 induce the phosphorylation of Cx43. Serum-starved 1007 and M10 cells were incubated for 5 min with 10 nM ET-1 or ET-3 in the absence or in the presence of BQ788 (100 nM). Cell lysates were immunoblotted with anti-Cx43 and with anti-β-actin for internal control.

B

C
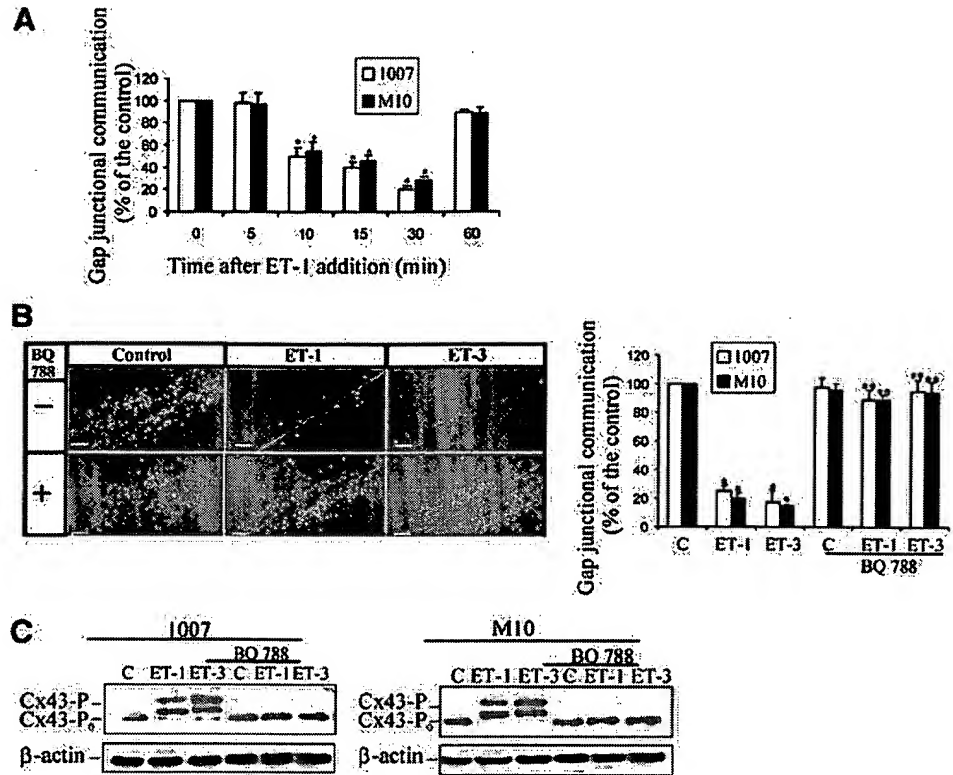
latent MMP-2, MMP-9, and MT1-MMP to their active form and TIMP-2 expression (Fig. 3*C*), as analyzed by Western blot and ELISA (Fig. 3, *B* and *C*). These data indicate that the activation of MMP, involved in a complex proteolytic cascade, is mediated by ET$_B$R activation.

ET-1 and ET-3 Increase $\alpha_2\beta_1$ and $\alpha_v\beta_3$ Integrin Expression. Because tumor cell invasiveness and metastasis formation depend on cell adhesive properties to the ECM (33–35), we investigated whether ETs were capable of modulating integrin expression on 1007 melanoma cells. Among the integrin subunits evaluated by flow cytometry, the levels of α_1, α_4, α_6, β_5, and β_6 integrin subunit expression in cells

stimulated with ET-1 and ET-3 (10 nM) for 24 h remained unaltered, whereas the levels of α_v, β_3, α_2, and β_1 expression were up-regulated (data not shown). Thus, in 1007 melanoma cells, ET-1 and ET-3 were able to significantly increase the heterodimeric $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrin expression (Fig. 4*A*), which play a major role in melanoma progression (33–35). These results were confirmed by Western blotting (Fig. 4*B*), demonstrating that both peptides significantly increased α_v, β_3, α_2, and β_1 expression that was blocked by BQ788. These results suggested that ETs through ET$_B$R activated $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins that could promote rapid adherence and increased motility of melanoma cells.

Fig. 3. ET-1 and ET-3 increase the secretion and activation of MMP-9, MMP-2, and MT1-MMP and the secretion of TIMP-2. *A*, serum-starved 1007 and M10 melanoma cell lines were stimulated for 24 h with 10 nM ET-1 or ET-3. Enzymatic activity of MMP-2 and MMP-9 was studied in conditioned media of melanoma cells by SDS-PAGE gelatin zymography, and gelatin lysis bands show migration positions of pro-MMPs and active MMPs. *B*, serum-starved 1007 cells were stimulated with 10 nM ET-1 or ET-3 for 24 h in the absence or presence of 100 nM BQ788. Conditioned media (MMP-2, MMP-9, and TIMP-2) or cell lysates (MT1-MMP) from melanoma cells were tested for the expression of MMP-9 (the M_r 92,000 proform and the M_r 76,000 active form), MMP-2 (the M_r 72,000 proform and M_r 64,000 active form), and MT1-MMP (the M_r 65,000 proform and the M_r 63,000 active form) and for TIMP-2 (M_r 21,000) by Western blotting. *C*, MMP-2 and TIMP-2 were also measured in conditioned medium from 1007 cells treated with 10 nM ET-1 or ET-3 in the absence or presence of BQ788 (100 nM) for 24 h using ELISA kit. Data are represented as means of results from three experiments each performed in duplicate. *Bars* indicate ±SD. *, $P \leq 0.001$ compared with control; **, $P < 0.0001$ compared with ET-1 and ET-3.
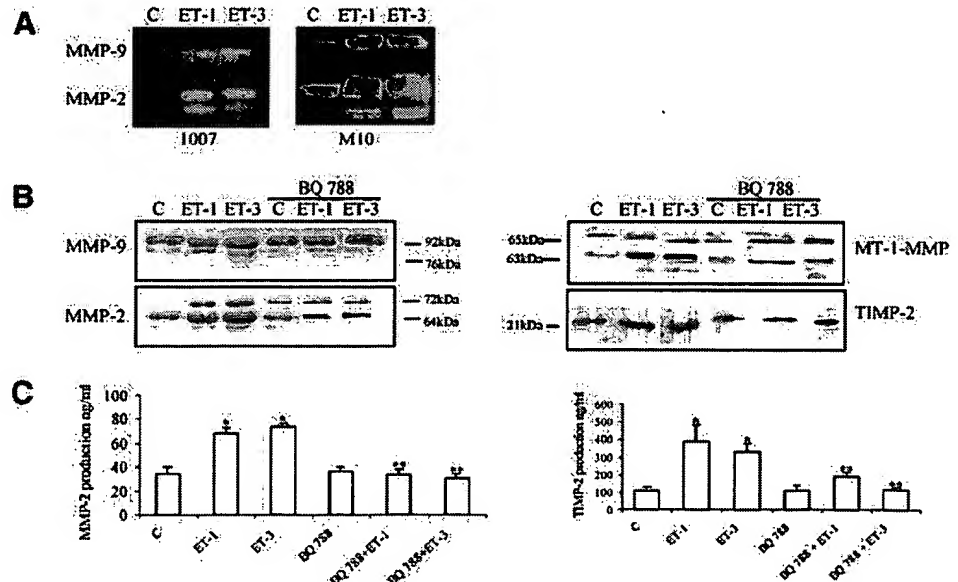
A

B

C

Fig. 4. ET-1 and ET-3 enhance $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrin expression. *A,* fluorescence-activated cell sorter analysis of $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrin expression by serum-starved 1007 melanoma cells treated with 10 nM ET-1 (*black*) or ET-3 (*gray*) for 24 h. *B,* serum-starved 1007 cells were treated with ET-1 or ET-3 (10 nM) in the absence or presence of BQ788 (100 nM). Cell lysates were analyzed by Western blot for the expression of α_2, β_1, α_v, and β_3 integrin subunits and for internal control with anti-β-actin. The relative density of integrin content was statistically analyzed and represents the average value of three independent Western blots ± SD; *, $P < 0.001$ compared with control; **, $P < 0.001$ compared with ET-1.
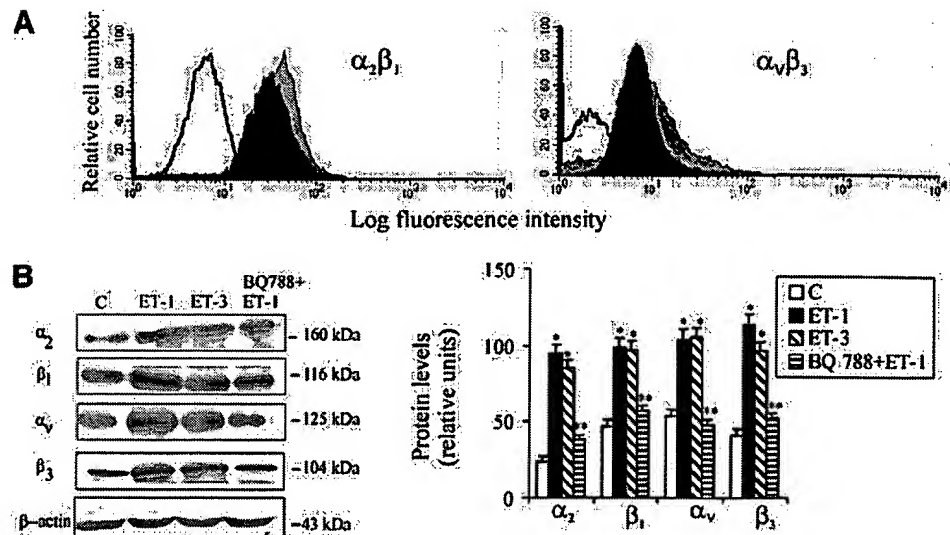
ET-1 and ET-3 Induce FAK and MAPK Activation through ET$_B$R.

Several lines of evidence have implicated that Erk and FAK pathways play a critical role in the oncogenic behavior of malignant melanoma (18–20). Because Erk and FAK activation represents a key event in ET-1-induced mitogenic signaling pathway (36), we investigated the effect of ETs on these ET$_B$R-mediated pathways in melanoma cells. ET-1 and ET-3 (10 nM) treatment resulted in a time-dependent induction of Erk1/2 and FAK phosphorylation, which reached a peak after 5 min and returned to baseline levels by 60 min (Fig. 5A). In presence of the constitutive activation of FAK and Erk1/2 in metastatic melanoma cell lines (18, 19), ET-1 and ET-3 were capable of increasing FAK and Erk1/2 activation in all four cell lines deriving from either primary (1007) or metastatic (M10, SKMel

28, and Mel120) tumors (Fig. 5B). The phosphorylation of Erk1/2 induced by ETs was inhibited by PD98059 (10 μM), a specific MAPK kinase inhibitor (Fig. 5B). To investigate which receptor subtype mediates FAK and Erk1/2 phosphorylation, BQ123 (100 nM), a specific ET$_A$R antagonist, and BQ788 (100 nM) were used in the presence or in absence of ET-1 and ET-3 (10 nM). BQ788, but not BQ123, was able to completely block ET-1- and ET-3-induced FAK and Erk1/2 activation (Fig. 5C), indicating that these signaling pathways are mediated through ET$_B$R. Furthermore, by treating 1007 adherent cells with cytochalasin-D to disrupt actin filaments, a reduced level of FAK phosphorylation after ET-1 and ET-3 (10 nM) stimulation was observed (Fig. 5D), indicating that the integrity of the cytoskeleton is required for ET-1- and ET-3-induced FAK activation in melanoma cells.

Fig. 5. *A,* kinetics of ET-1- and ET-3-induced FAK and Erk phosphorylation. Serum-starved 1007 cells were incubated for the indicated times with ET-1 or ET-3 (10 nM). Analysis of FAK phosphorylation was performed by immunoprecipitation with anti-FAK and subsequent immunoblotting by an antiphosphotyrosine. Analysis of the phosphorylated form of Erk1/2 was performed by immunoblotting with anti-phospho-Erk1/2. The membranes were reprobed with the specific anti-Erk1/2 and anti-FAK to ensure equal amounts of proteins. *B,* serum-starved primary (1007) and metastatic (M10, SKMel 28, and Mel120) cell lines were incubated for 5 min with ET-1 or ET-3 (10 nM) and analyzed for FAK and Erk phosphorylation as described previously. Analysis of phosphorylated forms of Erk1/2 was performed also on melanoma cells preincubated with 10 μM PD98059 for 20 min after incubation with ET-1 (10 nM). *C,* ET$_B$R antagonist blocks ET-induced FAK and Erk phosphorylation. Serum-starved 1007 cells were pretreated for 15 min with BQ123 (100 nM) or BQ788 (100 nM) in the absence or presence of 10 nM ET-1 or ET-3 for 5 min and then analyzed for FAK and Erk1/2 phosphorylation. The membranes were reprobed with the specific anti-Erk1/2 and anti-FAK to ensure equal amounts of proteins. *D,* ET-induced FAK phosphorylation is regulated by the cytoskeleton. Adherent 1007 cells, untreated or treated with 0.5 mg/ml cytochalasin-D (*Cyto D*), were stimulated with ET-1 or ET-3 (10 nM) for 5 min and then analyzed for FAK phosphorylation. The membranes were reprobed with the specific anti-FAK to ensure equal amounts of proteins.
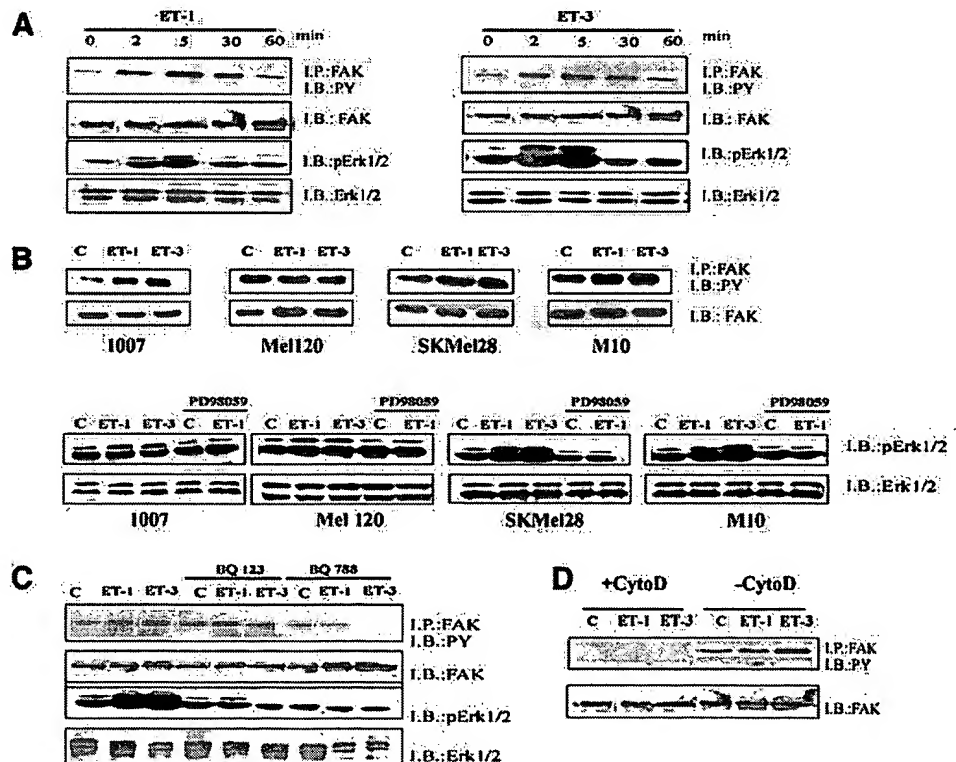
ET-1 and ET-3 Induce Melanoma Cell Proliferation, Adhesion, Migration, and Invasion through ET$_B$R. To examine the effects of ETs on cell proliferation, primary (1007) and metastatic (M10, SK-Mel28, and Mel120) cell lines were treated with different concentrations of ET-1 and ET-3. As shown in Fig. 6A, a significant and dose-dependent increase in [^{3}H]thymidine incorporation was observed in all melanoma cell lines tested.

To assess the effect of ET-1 and ET-3 on cell adhesion, 1007 cells were cultured with ET-1 and ET-3 for 24 h, and adhesion to type I collagen-coated surfaces was measured. A significant increase in cell adhesion was observed in ET-1- and ET-3-treated cells relative to control (Fig. 6B). We therefore examined the effect of ET-1 and ET-3 on the functional consequences of enhanced integrin expression and MMP activity on cellular events associated with metastatic spreading. Addition of 1 up to 100 nM ET-1 and ET-3 to melanoma cells induced a marked and dose-dependent increase in cell motility (Fig. 6C) and invasiveness (Fig. 6D). We investigated the ability of anti-$\alpha_2\beta_1$ and anti-$\alpha_v\beta_3$ Abs to interfere with adhesion and migration of 1007 melanoma cells. Abs to the $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins strongly reduced adhesion to type I collagen, as well as cell migration induced by ETs. Moreover, ET-1-stimulated-invasion was reduced to the control level in the presence of a potent chemical broad-spectrum MMP inhibitor, such as Ilomastat (10 μM), and in the presence of MEK inhibitor, such as PD98059 (10 μM), demonstrating that ETs are able to induce MMP-dependent invasion through MAPK signaling pathways. The stimulatory effect of 10 nM ET-1 and ET-3 on 1007 cell proliferation, adhesion, migration, and invasion was completely blocked by BQ788, whereas BQ123 only partially inhibited ET-induced effects, indicating ET$_B$R as a relevant receptor in these events associated with melanoma progression.

A-192621, a Selective ET$_B$R Antagonist, Inhibits Cell Proliferation and Melanoma Growth in Nude Mice. On the basis of the previous findings, we investigated the effect of the potent A-192621, an orally active nonpeptide ET$_B$R antagonist (37), on the *in vitro* and *in vivo* growth of M10 cells, which rapidly grow in nude mice. M10 cell line was incubated with ETs and/or with A-192621. The ET-induced proliferation was significantly inhibited in the presence A-192621 (100 nM; $P \leq 0.01$; Fig. 7A). We translated these results into a model of nude mice xenografted with M10 melanoma cells. Treatment with A-192621 produced a 60% inhibition of M10 tumor growth with either low (10 mg/kg/day) or high (20 mg/kg/day) doses (Fig. 7B). A-192621 treatment was generally well tolerated with no signs of acute or delayed toxicity, even at the highest A-192621 dose. Comparison of time course of tumor growth curves by two-way ANOVA with group and time as variables showed that the group-by-time interaction for tumor growth was statistically significant ($P < 0.0001$). Furthermore, the tumor growth inhibition obtained with A-192621 persisted for up 3 weeks after the termination of the treatment.

DISCUSSION

Progression of cutaneous melanoma requires the accumulation of a variety of phenotypical alterations that free the transformed melanocytes from the control of the surrounding normal microenvironment and enable them to acquire a stable invasive phenotype (15). Due to the resistance of melanoma to current therapies, the identification of molecular mechanisms underlying local and metastatic growth is mandatory for the development of novel treatments. Here, we have investigated whether the G-protein-coupled receptor activated by ETs may represent a relevant therapeutic target in this malignancy.

Loss of interactions between tumor cells and the surrounding normal microenvironment characterizes the progression of a number of
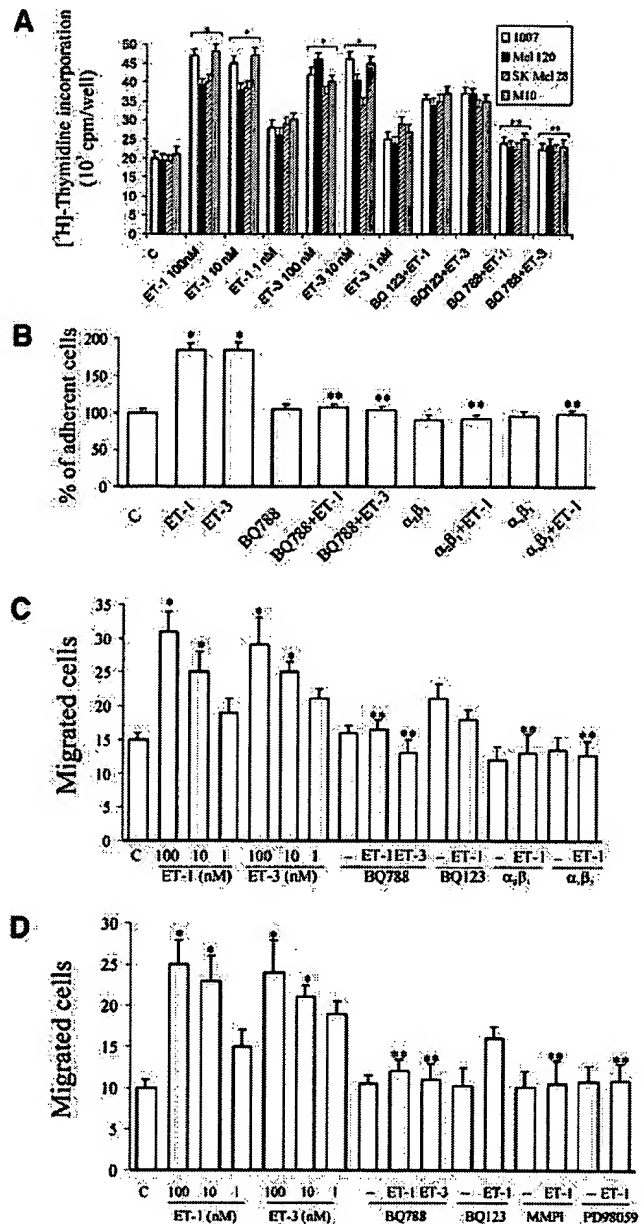

Fig. 6. Modulation of melanoma cell proliferation, adhesion, migration, and invasion by ET-1 and ET-3. A, ETs stimulate cell proliferation. Serum-starved 1007, M10, Mel120, and SKMel28 cells were treated with different concentrations of agonists for 24 h. Cells were treated with ET-1 or ET-3 (10 nM) in the absence or in the presence of BQ788 (100 nM) or BQ123 (100 nM) as indicated. *Bars* = mean ± SD of data from three independent experiments each performed in sextuplicate; *, $P \leq 0.0001$ compared with control; **, $P \leq 0.005$ compared with ET-1 or ET-3. B, ET-1 and ET-3 increase cellular adhesion to type I collagen. Serum-starved 1007 cells were cultured for 24 h with ET-1 or ET-3 (10 nM) in the absence or in the presence of BQ788 (100 nM). Inhibition of ET-1-stimulated 1007 cell adhesion to substrate by Abs $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrin (10 μg/ml) was analyzed. Data represent the means and SD from triplicate experiments each performed in sextuplicate. *, $P \leq 0.0001$ compared with control; **, $P \leq 0.005$ compared with ET-1 or ET-3. ET-1 and ET-3 induce melanoma cell migration (C) and invasion (D). Serum-starved 1007 cells (5 × 10^5 cells/ml) were treated with different doses of ET-1 or ET-3. Cells were treated with ET-1 or ET-3 (10 nM) in the absence or in the presence of BQ788 (100 nM) or BQ123 (100 nM), anti-$\alpha_2\beta_1$ and anti-$\alpha_v\beta_3$ integrins (10 μg/ml), MMP inhibitor (Ilomastat; 10 μM), or PD98059 (10 μM), as indicated. Data represent the means of results from three experiments each performed in triplicate. *, $P \leq 0.001$ compared with control. **, $P \leq 0.001$ compared with ET-1 or ET-3.

malignancies (17). Changes in cell surface adhesion molecules that modulate these interactions are likely to be a prerequisite for invasive growth. The shift in cadherin molecules from the E- to N-isotype has been correlated with tumor cell motility, invasiveness, and capability
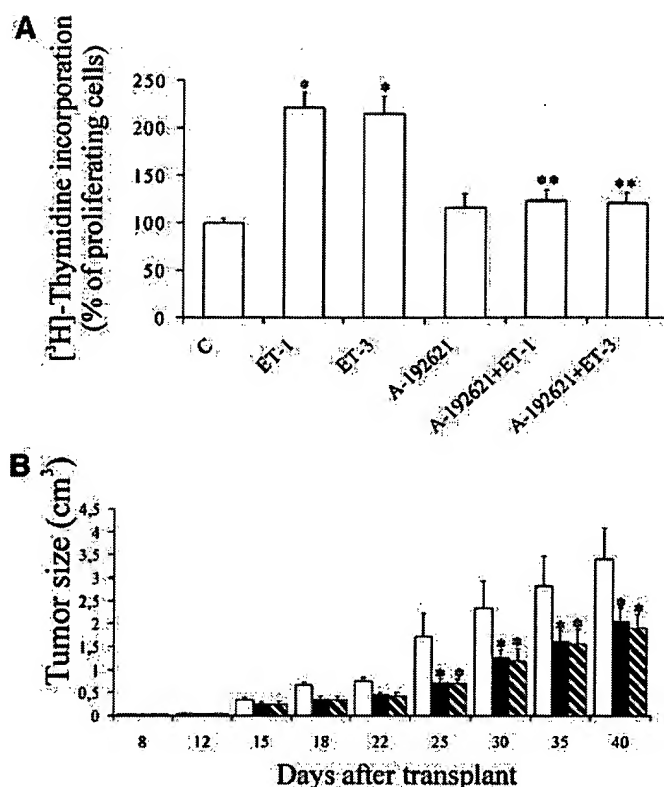
Fig. 7. A-192621, a selective ET$_B$R antagonist, inhibits cell proliferation and tumor growth *in vivo. A*, A-192621 blocks cell proliferation. Ten nM ET-1 or ET-3 was added to serum-starved M10 cells. A-192621 (100 nM) was incubated 15 min before the addition of ETs. Data are means of results from three experiments each performed in sextuplicate. *, $P \leq 0.001$ compared with control. **, $P \leq 0.01$ compared with ET-1 or ET-3. *B*, antitumor activity of ET$_B$R antagonist treatment on established M10 human melanoma xenografts. Mice received injection s.c. with 1.5×10^6 cells. Seven days after the tumor injection, mice were treated i.p. for 21 days with vehicle or with A-192621 (10 mg/kg/day, ■, or 20 mg/kg/day, ▨). Controls are indicated as □. Three different experiments were performed. Data represent the averages. *Bars* indicate ± SD. *, $P < 0.001$.

to interact with wider stromal counterparts in breast carcinoma (38). ET-1-induced down-regulation of E-cadherin, which is responsible for contact-mediated regulatory control by keratinocytes (16), has been described previously. Here, we demonstrate that ET-1 and ET-3 through ET$_B$R induce down-regulation of E-cadherin and associated catenin proteins, such as β-catenin and p120-catenin, with a parallel up-regulation of N-cadherin. This latter change allows homotypic adhesive contact as well as heterotypic (*i.e.*, fibroblasts, endothelial cells) melanoma cell-cell interactions (15).

Our results now document one pathway regulating transcription of E-cadherin. Consistent with the role of the transcriptional repressor *Snail* in melanoma (27), we demonstrate that ETs induce a significant up-regulation of *Snail* mRNA that concurs with the E-cadherin mRNA down-regulation. In addition, ETs can suppress the adherent junctional function by the tyrosine phosphorylation of β- and p120-catenins, resulting in increased levels of catenin-free pools that cause a decreased cell-cell adhesion.

Of interest, ETs appear to impair the gap junction communication system by inducing a transient and time-dependent reduction of GJIC and Cx43 phosphorylation, enabling melanoma cells to move into new cellular and stromal microenvironments (28).

Remodeling of the immediate ECM, which is a necessary step in tumor local invasion, requires the presence of active MMPs as well as their subcellular redistribution. With regard to melanoma, ET-1 can induce MMP secretion and activation as result of increased expression and activation of MT1-MMP and the concomitant up-regulation of

TIMP-2 (32). Melanoma cell invasiveness relies on a promigratory subset of cells at the tumor leading edge, which is characterized by a molecular program capable of silencing neighboring cells via cell-to-cell signaling and concomitant polarization of β1-integrin expression (39). Melanoma cells at the leading margin engage and cluster β1-integrins in anterior protrusions and show an increased expression and activity of MT1-MMP and MMP-2, leading to polarized ECM degradation and a collective movement of migration and invasion (15). The present study demonstrates that ETs are also capable of up-regulating β1 integrin, MT1-MMP, and MMP-2 expression, which is accompanied by increased rates of adhesion to ECM molecules and by increased migratory and invasive capacity. Moreover, in all of the melanoma cell lines tested ET-1 and ET-3 activated, through the ET$_B$R, FAK, and ERK, which are the signaling pathways claimed to contribute to the high metastatic potential of this tumor (18–20). Consistent with the role of ERK in regulating invasive activity, treatment of melanoma cells with the MEK inhibitor PD98059 reduced the MMP-dependent matrigel invasion of 1007 cells.

The present findings and a recent gene array profiling of melanoma has identified that ET$_B$R is a new marker associated with multiple aggressive phenotypes including the plasticity of melanoma cells to engage in vasculogenic mimicry (11). Screening a large series of benign and malignant pigment cell lesions from different progression stages using immunohistochemistry and quantitative reverse transcription-PCR analysis reveals ET$_B$R as a tumor progression marker in malignant melanoma and points to ET$_B$R as clinically relevant target especially in consideration of the development of small molecules capable of antagonizing ETs. Blockade of ET$_B$R by the peptide antagonist, BQ788, in fact has been demonstrated to inhibit melanoma cell growth *in vivo* and *in vitro* (14). In view of the potential use in clinical settings, we showed that the specific nonpeptidic orally active ET$_B$R antagonist A-192621 displays antitumor activity against established melanoma expressing ET$_B$R. In conclusion, we have identified multiple molecular pathways elicited by ET-1 and ET-3 that regulate melanoma local and metastatic growth. Because all of the molecular effectors involved in melanoma progression including cell-cell adhesion and cell-cell communication molecules, tumor proteases, and integrins are triggered by the ET$_B$R activity, blockade of this receptor by small molecules results in inhibition of melanoma growth *in vitro* and *in vivo*, thus offering the possibility of exploring targeted therapy in this malignancy. In view of known resistance of melanoma to current therapies, this knowledge may be of clinical relevance to assess the extent to which ET$_B$R blockade can be exploited in integrated treatments.

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REFERENCES

1. Shin, M. K., Levorse, J. M., Ingram, R. S., and Tilghman, S. M. The temporal requirement for endothelin receptor-B signalling during neural crest development. Nature (Lond.), *402:* 496–501, 1999.
2. Herlyn, M., Berking, C., Li, G., and Satyamoorthy, K. Lessons from melanocyte development for understanding the biological events in naevus and melanoma formation. Melanoma Res., *10:* 303–312, 2000.
3. Levin, E. R. Endothelins. N. Engl. J. Med., *333:* 356–363, 1995.
4. Reid, K., Turnley, A. M., Maxwell, G. D., Kurihara, Y., Kurihara, H., Bartlett, P. F., and Murphy, M. Multiple roles for endothelin in melanocyte development: regulation of progenitor number and stimulation of differentiation. Development, *122:* 3911–3919, 1996.

5. Scott, G., Cassidy, L., and Abdel-Malek, Z. α-Melanocyte-stimulating hormone and endothelin-1 have opposing effects on melanocyte adhesion, migration, and pp125FAK phosphorylation. Exp. Cell Res., *237:* 19–28, 1997.

6. Imokawa, G., Yada, Y., and Miyagishi, M. Endothelins secreted from human keratinocytes are intrinsic mitogens for human melanocytes. J. Biol. Chem., *267:* 24675–24680, 1992.

7. Tada, A., Suzuki, I., Im, S., Davis, M. B., Cornelius, J., Babcock, G., Nordlund, J. J., and Abdel-Malek, Z. A. Endothelin-1 is a paracrine growth factor that modulates melanogenesis of human melanocytes and participates in their responses to ultraviolet radiation. Cell Growth & Differ., *9:* 575–584, 1998.

8. Imokawa, G., Miyagishi, M., and Yada, Y. Endothelin-1 as a new melanogen: coordinated expression of its gene and the tyrosinase gene in UVB-exposed human epidermis. J. Invest. Dermatol., *105:* 32–37, 1995.

9. Nelson, J., Bagnato, A., Battistini, B., and Nisen, P. The endothelin axis: emerging role in cancer. Nat. Rev. Cancer, *3:* 110–116, 2003.

10. Yohn, J. J., Smith, C., Stevens, T., Hoffman, T. A., Morelli, J. G., Hurt, D. L., Yanagisawa, M., Kane, M. A., and Zamora, M. R. Human melanoma cells express functional endothelin-1 receptors. Biochem. Biophys. Res. Commun., *201:* 449–457, 1994.

11. Bittner, M., Meltzer, P., Chen, Y., Jiang, Y., Seftor, E., Hendrix, M., Radmacher, M., Simon, R., Yakhini, Z., Ben-Dor, A., *et al.* Molecular classification of cutaneous malignant melanoma by gene expression profiling. Nature (Lond.), *406:* 536–540, 2000.

12. Demunter, A., De Wolf-Peeters, C., Degreef, H., Stas, M., and van den Oord, J. J. Expression of the endothelin-B receptor in pigment cell lesions of the skin: evidence for its role as tumor progression marker in malignant melanoma. Virchows Arch., *438:* 485–491, 2001.

13. Eberle, J., Fecker, L. F., Orfanos, C. E., and Geilen, C. C. Endothelin-1 decreases basic apoptotic rates in human melanoma cell lines. J. Invest. Dermatol., *119:* 549–555, 2002.

14. Lahav, R., Heffner, G., and Patterson, P. H. An endothelin receptor B antagonist inhibits growth and induces cell death in human melanoma cells *in vitro* and *in vivo.* Proc. Natl. Acad. Sci. USA, *96:* 11496–11500, 1999.

15. Li, G., Satyamoorthy, K., Meier, F., Berking, C., Bogenrieder, T., and Herlyn, M. Function and regulation of melanoma-stromal fibroblast interactions: when seeds meet soil. Oncogene, *22:* 3162–3171, 2003.

16. Hsu, M., Andl, T., Li, G., Meinkoth, J. L., and Herlyn, M. Cadherin repertoire determines partner-specific gap junctional communication during melanoma progression. J. Cell Sci., *113:* 1535–1542, 2000.

17. Ruiter, D., Bogenrieder, T., Elder, D., and Herlyn, M. Melanoma-stroma interactions: structural and functional aspects. Lancet Oncol., *3:* 35–43, 2002.

18. Kahana, O., Micksche, M., Witz, I. P., and Yron, I. The focal adhesion kinase (P125FAK) is constitutively active in human malignant melanoma. Oncogene, *21:* 3969–3977, 2002.

19. Satyamoorthy, K., Li, G., Gerrero, M. R., Brose, M. S., Volpe, P., Weber, L. B., van Belle, P., Elder, D. E., and Herlyn, M. Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. Cancer Res., *63:* 756–759, 2003.

20. Smalley, K. S. M. A pivotal role for ERK in the oncogenic behaviour of malignant melanoma. Int. J. Cancer, *104:* 527–532, 2003.

21. Remuzzi, G., Perico, N., and Benigni, A. New therapeutics that antagonize endothelin: promises and frustrations. Nat. Rev. Drug Discovery, *1:* 986–1001, 2002.

22. Golub, S. H., Hanson, D. C., Morton, D. L., Pellegrino, M. A., Sulit, H. L., and Ferrone, S. Comparison of histocompatibility antigens on cultured human tumor cells and fibroblasts by quantitative antibody absorption and sensitivity to cell-mediated cytotoxicity. J. Natl. Cancer Inst. (Bethesda), *58:* 167–171, 1976.

23. Bagnato, A., Salani, D., Di Castro, V., Wu-Wong, J. R., Tecce, R., Nicotra, M. R., Venuti, A., and Natali, P. G. Expression of endothelin-1 and endothelin A receptor in ovarian carcinoma: evidence for an autocrine role in tumor growth. Cancer Res., *59:* 720–727, 1999.

24. Rosanò, L., Varmi, M., Salani, D., Di Castro, V., Spinella, F., Natali, P. G., and Bagnato, A. Endothelin-1 induces tumor proteinase activation and invasiveness of ovarian carcinoma cells. Cancer Res., *61:* 8340–8346, 2001.

25. Hsu, M. Y., Meier, F. E., Nesbit, M., Hsu, J. Y., Van Belle, P., Elder, D. E., and Herlyn, M. E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors. Am. J. Pathol., *156:* 1515–1525, 2000.

26. Jamal, S., and Schneider, R. J. UV-induction of keratinocyte endothelin-1 downregulates E-cadherin in melanocytes and melanoma cells. J. Clin. Investig., *110:* 443–452, 2002.

27. Poser, I., Dominguez, D., Garcia de Herreros, G., Varnai, A., Buettner, R., and Bosserhoff, A. K. Loss of E-cadherin expression in melanoma cells involves upregulation of the transcriptional repressor Snail. J. Biol. Chem., *276:* 24661–24666, 2001.

28. Muller, T., Choida, A., Reichmann, E., and Ullrich, A. Phosphorylation and free pool of β-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration. J. Biol. Chem., *274:* 10173–10183, 1999.

29. Rivedal, E., and Opsahl, H. Role of PKC and MAP kinase in EGF- and TPA-induced connexin43 phosphorylation and inhibition of gap junction intercellular communication in rat liver epithelial cells. Carcinogenesis, *22:* 1543–1550, 2001.

30. Spinella, F., Rosanò, L., Di Castro, V., Nicotra, M. R., Natali, P. G., and Bagnato, A. Endothelin-1 decreases gap junctional intercellular communication by inducing phosphorylation of connexin 43 in human ovarian carcinoma cells. J. Biol. Chem., *278:* 41294–41301, 2003.

31. Brinckerhoff, C. E., and Matrisian, L. M. Matrix metalloproteinases: a tail of a frog became a prince. Nat. Rev. Mol. Cell. Biol., *3:* 207–214, 2002.

32. Bernardo, M. M., and Fridman, R. TIMP-2 (tissue inhibitor of metalloproteinase-2) regulates MMP-2 (matrix metalloproteinase-2) activity in the extracellular environment after pro-MMP-2 activation by MT1 (membrane type1)-MMP. Biochem. J., *374:* 739–745, 2003.

33. Natali, P. G., Nicotra, M. R., Bartolazzi, A., Cavaliere, R., and Bigotti, A. Integrin expression in cutaneous malignant melanoma: association of the α3β1 heterodimer with tumor progression. Int. J. Cancer, *54:* 68–72, 1993.

34. Natali, P. G., Hamby, C. V., Felding-Habermann, B., Liang, B., Nicotra, M. R., Di Filippo, F., Giannarelli, D., Temponi, M., and Ferrone, S. Clinical significance of α(v)β3 integrin and intercellular adhesion molecule-1 expression in cutaneous malignant melanoma lesions. Cancer Res., *57:* 1554–1560, 1997.

35. Eguchi, H., and Horikoshi, T. The expression of integrin α2 β1 and attachment to type I collagen of melanoma cells are preferentially induced by tumour promoter, TPA (12-*O*-tetradecanoyl phorbol-13-acetate). Br. J. Dermatol., *134:* 33–39, 1996.

36. Bagnato, A., Tecce, R., Di Castro, V., and Catt, K. J. Activation of mitogenic signaling by endothelin-1 in ovarian carcinoma cells. Cancer Res., *57:* 1306–1311, 1997.

37. Griswold, D. E., Douglas, S. A., Martin, L. D., Davis, T. G., Davis, L., Ao, Z., Luttmann, M. A., Pullen, M., Nambi, P., Hay, D. W., *et al.* Endothelin B receptor modulates inflammatory pain and cutaneous inflammation. Mol. Pharmacol., *56:* 807–812, 1999.

38. Hazan, R. B., Phillips, G. R., Qiao, R. F., Norton, L., and Aaronson, S. A. Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J. Cell. Biol., *148:* 779–790, 2000.

39. Hegerfeldt, Y., Tusch, M., Brocker, E. B., and Friedl, P. Collective cell movement in primary melanoma explants: plasticity of cell-cell interaction, β1-integrin function, and migration strategies. Cancer Res., *62:* 2125–2130, 2002.